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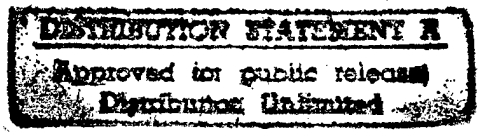
Japan

BIO JAPAN '92 SYMPOSIUM PROCEEDINGS



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43070028E Tokyo BIO JAPAN '92 SYMPOSIUM PROCEEDINGS in English 26-28 Aug 92 pp various

[Selected papers from BIO JAPAN '92 SYMPOSIUM PROCEEDINGS organized by Japan Bioindustry Association (JBA) and co-organized by Japan Health Sciences Foundation (JHSF), Society for Techno-Innovation of Agriculture, Forestry and Fisheries (STAFF)]

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NEURAL ACTIVITY AND GENE EXPRESSION IN THE HIPPOCAMPUS

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ABSTRACT

In situ hybridization and immunohistochemistry were performed on the rat hippocampus to investigate the changes in gene expression induced by kainic acid and other convulsants. The cRNA probes and antibodies were for immeidate early genes (c-fos and Zif268), neuropeptides (somatostatin, proenkephalin and cholecystokinin) and neuronal cytoskeletal protein (neurofilament L subunit). The findings on kainate-induced convulsion are as follows: c-fos and Zif268 were expressed in most hippocampal neurons but their maintenance corresponded to the duration of the seizures. Somatostatin is expressed in interneurons of the control animals but appeared in principal neurons, namely hippocampal pyramidal cells and dentate granule cells. When the seizure was interrupted with diazepam, somatostatin mRNA did not appeared in granule cells. Kainate remarkably potentiated the expression of proenkephalin in granule cells and of cholecystokinin in CA1 pyramidal cells. Unexpectedly, neurofilament L-mRNA was increased by kainate.

Recently stimulus-transcription coupling(1) has attracted the attention of neuroscience. Some long-lasting changes in function and structure of the nervous system are regarded as the bases of learning and memory. Therefore, activation of nerve cells by various kinds of stimuli not only induces rapid responses for signal transmission to the other neurons but also leaves any persistent changes which will affect the subsequent neuronal responses. The latter properties of the nervous system are called neural plasticity and the long-term changes lasting for weeks and years will be maintained by altered gene expression followed by protein synthesis.

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Brilliant progress in molecular biology has permitted us to identify and localize biologically active molecules in the cells. This approach is particularly suitable for cellular and chemical analyses of the nervous tissue which consists of heterogeneous neurons with different connections and functions. Differential screening of cDNA libraries using control and experimental probes or screening of expression cDNA library with monoclonal antibodies of interest is expected to isolate any brain function-specific cDNA clones. We have tried to obtain cDNA clones by differential screening of subtracted cDNA library from the rat hippocampus after kindling, lifelong seizure susceptibility acquired after repetition of ineffective stimuli(2). Quantification of mRNA after filter hybridization an dlocalization of mRNA with in situ hybridization histochemistry are used to correlate the change in gene expression with neural activity.

Furthermore, invesaitgations have now been concentrated for disclosing the regulatory mechanism of gene expression. Interaction of transcription factor(s) with each gene initiates expression of the gene. For this, phosphorylation or other modification of existing transcription factors or de novo synthesis of other transcription factors is required. Examples of the former group of transcription factors are cyclicAMP response element-binding protein (CREB) and serum response factor (SRF) and those of the latter group are c-Fos, c-Jun and Zif268. The latter genes are included also in immediate early genes (IEGs), whose expression is activated rapidly by growth factors and other stimuli, and does not need de novo synthesis of other regulatory proteins.

In 1986 the first demonstration was made on casual relationship between neural activity and gene expression. High potassium-induced depolarization(3) and receptor activation by nicotine(4) produced a rapid (within 5 miniutes) and transient expression of c-fos in PC12 cells. Subsequently many papers have been published on expression of c-fos and other IEGs in the brain after physiological and non-physiological stimulation. On the other hand, expression of enkephalin and endorphin is discussed on their involvement in pain and convulsion. A study with a transgenic mouse containing a fusion gene showed some regional and seizure-type specificity of c-fos expression (5). However, the situation is still controversial and few is known on the casual relationship of expression between transcription factor-coding IEGs and their target genes encoding the functional neuron-specific proteins.

In the present paper we studied the expression of IEGs(c-fos and zif268), neuropeptides(somatostatin, proenkephalin and cholecystokinin) and intermediate filament protein (neurofilament protein L subunit) in the rat hippocampus and other brain areas with in situ hybridization and also performed immunohistochemistry against their products(6-8). The distribution of positive neurons was compared between normal and convulsive rats. Convulsion was induced mainly by kainic acid and in some rats by pentetrazole and picrotoxin. Slice preparation was prepared from the rat hippocampus and subjected to conventional electrophysiology. Then in situ hybridization was performed on thin sections of the slice to correlate with its electrical activity(9, 10). Digoxigenin-labeled antisense cRNAs were prepared for in situ hybridization. They were detected histochemically after

binding with alkaline phosphatase-labeled anti-digixigenin antibody. Immunohistochemical detection was made by ABC method.

Results of in situ hybridization on kainate-induced convulsions are described here. Subcutaneous injection of adult rats(Wistar) with 8.5 mg/kg body weight of kainic acid induced so-called limbic seizure. After frequent "wet dog shake" behavior, generalized convulsion occurred intermittently around 1.5 hours after the administration and then turned to the status epilepticus which lasted for several hours. Duration of seizure was controlled by administration of diazepam (10 mg/kg). The animals were sacrificed at 1.5-48 hours after kainate injection and the brains were excised for preparation of frozen sections.

c-fos and zif268: In the control hippocampus no c-fos expression was observed but zif268 were expressed in several cells. After kainate injection all neurons expressed c-fos and zif268. Exact time relation with onset of the seizure was not known but the maximal expression was attained within 1.5 hours. The expression was transient and its duration depended on the duration of seizure. c-fos expression in the hippocampal subfields and cortex after long-lasting seizures was shown with that of somatostatin in Fig. 1.

Somatostatin (preprosomatostatin): In the control hippocampus both somatostatin mRNA and peptide are present only in some interneurons in the dentate hilus and hippocampal formation. After kainate a subpopulation of principal neurons in the hippocampus, namely dentate granule cells and pyramidal cells, expressed somatostatin. This induction started at 3 hours and reached a maximum at 8 hours after kainate. When the seizure was suppressed early by diazepam, the induction was restricted to pyramidal cells. Somatostatin is expressed transiently in several types of neurons during development, but hippocampal granule and pyramidal cells are not included in this group. No papers appears to have reported the transient activity-dependent expression of somatostatin as observe here. Physiological functions of somatostatin in nerve cells are not known exactly, but somatostatin depresses the firing of some neurons and promotes neurite growth in cell culture. Therefore, it might be possible that somatostatin prevents excessive activation of neurons during the seizure and promotes sprouting of the nerve terminals afterward.

Enkephalin (preproenkephalin) and cholecystokinin: Originally enkephalin is expressed in dentate granule cells and cholecystokinin is observed in CA1 pyramidal cells and interneurons. After kainate seizure these expression in principal neurons was potentiated markedly. For example, about 15 percent of granule cell were expressing preproenkephalin mRNA moderately in the control but after kainate-induced seizure all granule cells expressed it to the maximal extent. Increase in enkephalin expression and decrease in dynorphin expression appear to be a general change after various kinds of convulsions(11).

Neurofilament protein L subunit (NF-L): At the beginning we expected that NF-L mRNA would not be changed by neural activity and serve as a reference when other mRNAs were affected. Surprisingly, NF-L mRNA in granule cells were increased

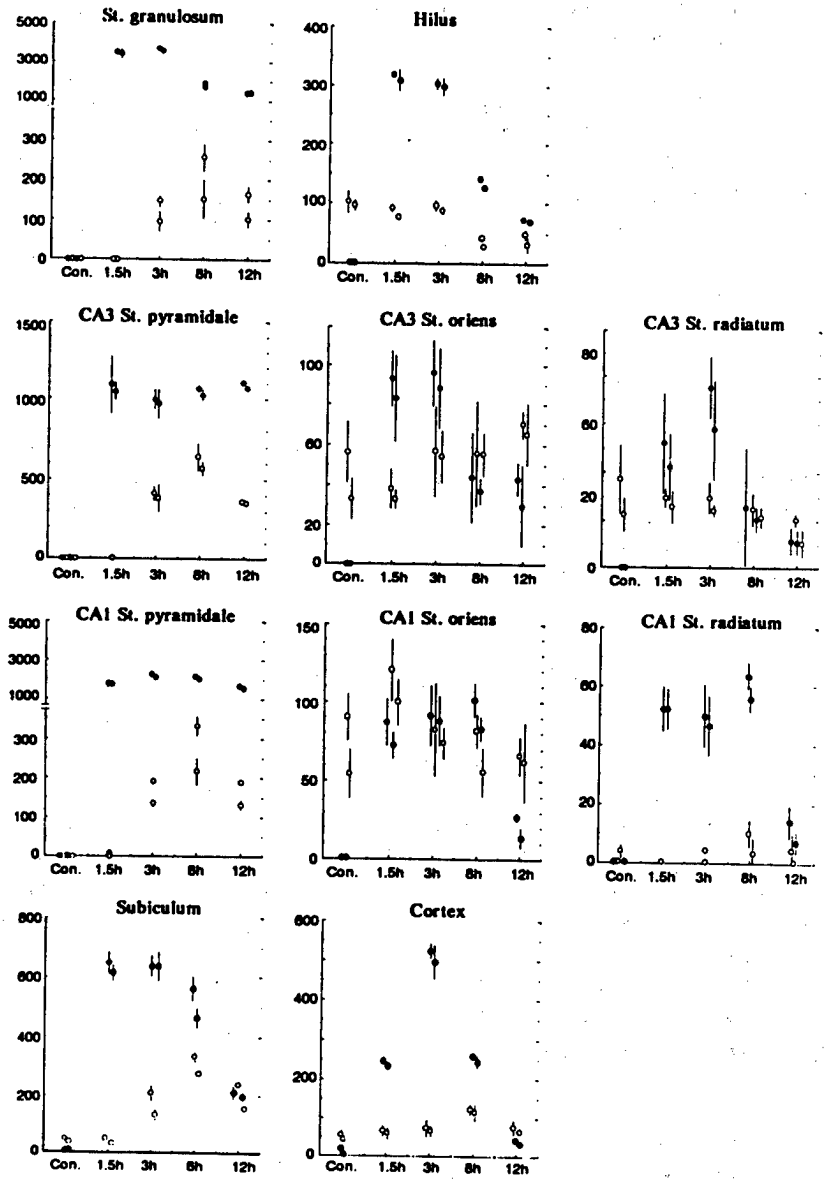

Fig. 1. Kainic acid-induced changes in densities of c-fos mRNA(●) and prepro-somatostatin mRNA(○)-expressing cells in the hippocampus and cerebral cortex. Abscissae, time after injection of kainic acid. Ordinates, positive cell density (cells/square mm). From ref.7.

as early as 1.5 hours after kainate injection. We are now studying the intracellular distribution of NF-L protein and the mechanism of this gene expression.

Pentetrazole and picrotoxin: These convulsants act with different mechanism and, therefore, will activate neurons affecting their gene expression differently from kainic acid. Patterns of c-fos expression in the hippocampus were different between kainate and pentetrazole. Changes in gene expression as observed for kainate were not remarkable for picrotoxin but a noticeable finding is an increase in tyrosine hydroxylase mRNA in the substantia nigra (O. Stork and K.O., unpublished).

Use of slice preparation: Electrical activity of the neural network in brain slice preparation can be maintained up to half a day. It can be recorded with conventional elecrophysiological techniques but also by a combination of voltage sensitive dye and constantly-advancing optical recording techniques. Therefore, when in situ hybridization can be performed on the slice preparation after manipulation of neural activity and its recording, the exact correlation will be obtained at cellular level. We first found that the hippoicampal slices conventionally obtained from unanesthetized rats expressed IEGs transiently around 1 hours after decapitation. This expression was partly prevented by deep anesthesia with Nembutal. When the slices were incubated in artificial solution (Krebs-Ringer solution), their electrical activity were maintained constant but some mRNAs, such as those for proenkephalin and NF-L, continued to be decreased. Supplementation with vitamin and amino acid mixture for tissue culture medium fairly improved the mRNA level. Somatostatin mRNA of interneurons was maintained well in Krebs solution.

Kainic acid induced excessive firing of most neurons and then suppressed the electrically-evoked responses for 30-60 minutes. In these slices c-fos expression was observed in all neurons. When a low concentration (0.1 uM) of kainic acid was applied for several minutes, intermittent bursting discharges occurred spontaneously for hours. The origin of this bursting was localized at CA1 region. In situ hybridization demonstrated that c-fos expression was localized in CA1 pyramidal cells.

Investigations of stimulus-induced gene expression have just started. Future extensive analyses, introducing up-to-date molecular and cellular engineering techniques, are expected to disclose the molecular mechanism of neural functions including plasticity.

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A-2-4

PROBLEMS IN PRODUCING GLYCOPROTEINS BY GENE-TECHNOLOGY

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ABSTRACT

Many proteins produced in living organisms contain sugars. Recent advancement of cell biology has revealed that these sugar chains play important roles as signals of cell surface recognition phenomena. This review will give an outline of the structural characteristics and the biosynthetic mechanism of the *N*-linked sugar chains of glycoproteins to help readers to consider the problems which will be encountered in producing glycoproteins by recombinant technique.

INTRODUCTION

The first epoch of glycoconjugate research came in late 1950th, when the structures of human ABO and Lewis blood group determinants and the molecular basis of the antigenic alteration of bacteria were elucidated. Another new wave came after 1980th. Development in cell biology suggested that the sugar chains of glycoconjugates play important roles as the signals of various cellular recognitions, which are important in fertilization, embryogenesis, and organ formation. Furthermore, recent development of gene-technology strongly accelerated the expansion of glycoconjugate research. By the establishment of the new bio-technology, many minor but important proteins found in living organisms could be obtained in large amount with use of microorganisms. However, many proteins produced by animal cells occur as glycoproteins. Because microorganisms such as *E. coli* lack glycosylation machinery, recombinant proteins produced by the bacteria lack sugar chains. Since many of these non-glycosylated proteins do not express expected biological activities, the functional role of the sugar chains of glycoproteins has attracted researchers attention. Based on an idea to elucidate the biological information included in the sugar chains of glycoproteins and other glycoconjugates, and introduce them as knowledge of biology, a novel scientific field called "glycobiology" has been established recently.
In this symposium, I would like to give an outline of this newly developing bioscience field, and help readers to solve various problems in producing glycoproteins by recombinant technique.

I. STRUCTURAL RULES INCLUDED IN THE *N*-LINKED SUGAR CHAINS OF GLYCOPROTEINS.

The sugar chains most abundantly detected in glycoproteins are classified into two groups. *O*-Linked sugar chains or mucin type sugar chains contain an *N*-acetylgalactosamine residue at their reducing termini. This *N*-acetylgalactosamine residue is linked to the hydroxyl group of either serine or threonine residue of polypeptide by α-anomeric linkage. *N*-Linked sugar chains or asparagine-linked sugar chains contain *N*-acetylglucosamine group at their reducing termini, which is then linked to the amide group of asparagine residue of polypeptide by β-anomeric linkage.

All *N*-linked sugar chains contain the pentasaccharide: Manα1→6(Manα 1→3)Manβ1→4GlcNAcβ1→4GlcNAc as the common core structure. This pentasaccharide is called "trimannosyl core". They are further classified into three subgroups (Fig. 1). Sugar chains, which fall into the first group, contain only α-mannosyl residues linked to the common trimannosyl core and are called high mannose type sugar chains. A heptasaccharide with two branching structures: Manα 1→6(Manα1→3)Manα1→6(Manα 1→3)Manβ1→4GlcNAcβ1→4GlcNAc is commonly included in this type of sugar chains as shown by dotted line in Fig. 1. Variation is formed in these sugar chains by the numbers and the locations of up to four Manα1→2 residues linked to the three non-reducing terminal α-mannosyl residues of the common heptasaccharide. The sugar chains which fall into the second group, complex type sugar chains, contain no other mannosyl residue than the trimannosyl core. Instead, outer chains with *N*-acetylglucosamine residue at their reducing termini are linked to the two α-mannosyl residues of the trimannosyl core. These outer chains are composed of *N*-acetylglucosamine, galactose, fucose, sialic acid, *N*-acetylgalactosamine and sulfate. Presence or absence of the α-fucosyl residue linked to the C-6 position of proximal *N*-acetylglucosamine and the β-*N*-acetylglucosamine residue linked to the C-4 position of the β-mannosyl residue of the trimannosyl core (bisecting GlcNAc) contributes the structural variation of the complex type sugar chains. The third group is called hybrid type[1], because the oligosaccharides which fall into this group have the structural features of both high mannose type and complex type sugar chains. One or two α-mannosyl residues are linked to the Manα1→6 arm of the trimannosyl core like in the case of high mannose type, and the outer chains found in complex type sugar chains are linked to the Manα1→3 arm of the core of this group. Presence or absence of the α-fucosyl residue and the bisecting GlcNAc linked to the trimannosyl core also induce structural variation of the sugar chains of this subgroup. Among the three subgroups of *N*-linked sugar chains, complex type has the largest structural variation. This variation is formed by two structural factors. From one to five outer chains are linked to the trimannosyl core by different linkages, resulting in formation of mono-, bi-, tri-, tetra- and pentaantennary sugar chains. Two isomeric tri-

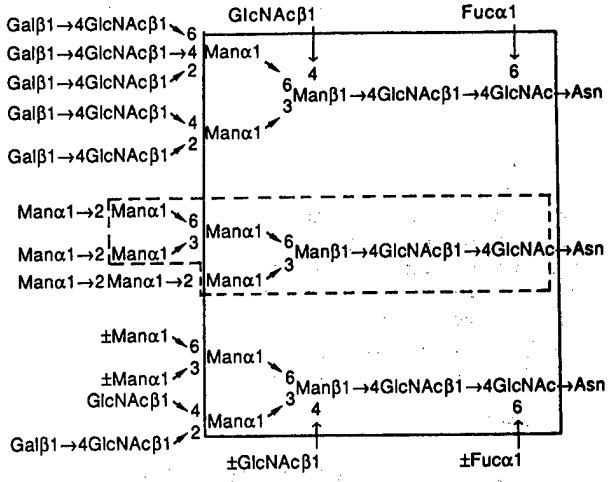

Fig. 1. Three subgroups of *N*-linked sugar chains.

antennary sugar chains containing either the GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3 group or the GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6 group are found. These isomeric triantennary sugar chains are called 2,4-branched and 2,6-branched triantennary sugar chains, respectively. Various structures are found for the outer chain moieties of complex type sugar chains. Combination of antennary and the various outer chains will form a large number of different complex type sugar chains.
In contrast to *N*-linked sugar chains, *O*-linked sugar chains contain less structural rules.

II. BIOSYNTHESIS OF *N*-LINKED SUGAR CHAINS —— A UNIQUE PATHWAY INCLUDING THE PROCESSING AND THE MATURATION.

N-Linked sugar chains are formed by a series of complex pathway including lipid-linked intermediates[2]. First, GlcNAc-1-P is transfered from UDP-GlcNAc to a polyisoprenol monophosphate:dolichyl phosphate (Dol-P). The *N*-acetylglucosamine residue of the GlcNAc-P-P-Dol is the sprout of *N*-linked sugar chain. To this *N*-acetylglucosamine residue, another *N*-acetylglucosamine and five mannose residues are transfered from UDP-GlcNAc and GDP-Man, respectively. The lipid bound heptasaccharide is converted to $Glc_3{\cdot}Man_9{\cdot}GlcNAc_2$-P-P-Dol by further addition of four α-mannosyl residues from Dol-P-Man and three α-glucosyl residues from Dol-P-Glc. The tetradecasaccharide of the lipid derivative is then transferred *en bloc* to the asparagine residue of the polypeptide chain, which is being translated in rough endoplasmic reticulum by the catalytic action of dolichylpyrophosphoryl oligosaccharide:polypeptide oligosaccharyltransferase residing in the endoplasmic membrane. Only the asparagine residues in the sequence of Asn-X-Ser/Thr, where X can be any amino acid other than proline, are glycosylated.
The completely translated polypeptide with the tetradecasaccharide is then transported to Golgi apparatus. During this transport, three α-glucosyl residues and at least one Manα1→2 residue are removed by the action of two α-glucosidases and an α-mannosidase residing in the membrane of endoplasmic reticulum. After being translocated to *cis*-Golgi, the *N*-linked sugar chain of the polypeptide is converted to $Man_5{\cdot}GlcNAc_2$ by the action of Golgi α-mannosidase I which removes all Manα1→2 residues from the sugar chain. A series of high mannose type sugar chains is now considered as the intermediary product of this trimming process. When the glycoprotein is translocated to *medial*-Golgi, an *N*-acetylglucosamine residue is added at the C-2 position of the Manα1→3 arm of the trimannosyl core portion by the action of *N*-acetylglucosaminyltransferase I (GnT-I). Addition of this *N*-acetylglucosamine residue changes the sterical arrangement of the two α-mannosyl residues, so that they can be removed by Golgi α-mannosidase II. These are the whole features of the processing pathway to form monoantennary complex type sugar chain. Action of another *N*-acetylglucosaminyltransferase (GnT-III) to the processing intermediates converts them to bisected sugar chains. The α-mannosyl residues of these bisected sugar chains cannot be removed by Golgi α-mannosidase II. Hybrid type sugar chains are considered to be produced from these deadlocked intermediates.
Starting from monoantennary sugar chains, a series of complex type sugar chains is formed by the action of various *N*-acetylglucosaminyltransferases[3] (Fig. 2). Each β-*N*-acetylglucosamine residue is further elongated by the action of various glycosyltransferases as depicted in Fig. 3. Bisecting GlcNAc residue cannot be the acceptor of any of these glycosyltransferases, and remains unchanged during these maturation processes. Glycosyltransferases catalyzing these reactions have strict specificities for donor and acceptor substrates. Under the cellular

condition where enough nucleotide sugar pools are available, levels and acceptor specificities of glycosyltransferases are of primary importance for determining the final structure of an outer chain moiety of complex type sugar chain. For example, an α2,6-sialyltransferase purified from rat liver can transfer sialic acid to the Galβ1→4GlcNAc group producing the Siaα2→6Galβ1→4GlcNAc group, but not to the Galβ1→3GlcNAc group. An α2,3-sialyltransferase purified from the same source can transfer sialic acid to both the Galβ1→4GlcNAc and the Galβ1→3GlcNAc groups[4]. Therefore, an oligosaccharide structure can be recognized by a glycosyltransferase or by some glycosyltransferases. In the latter case, distinct glycosyl linkages are formed in a different ratio as a result of competition between glycosyltransferases. Competition also occurs between glycosyltransferases which add different sugars to a common acceptor sugar chain. A typical example is the case of sialylation and fucosylation of the Galβ1→4GlcNAc group in the complex type sugar chains. An α2,6-sialyltransferase purified from bovine colostrum and an α1,3-fucosyltransferase from human milk can produce the Siaα2→6Galβ1→4GlcNAc and the Galβ1→4(Fucα1→3)GlcNAc groups by acting on asialotransferrin, respectively[5]. However, fucosylated asialotransferrin cannot serve as a substrate for the α2,6-sialyltransferase.

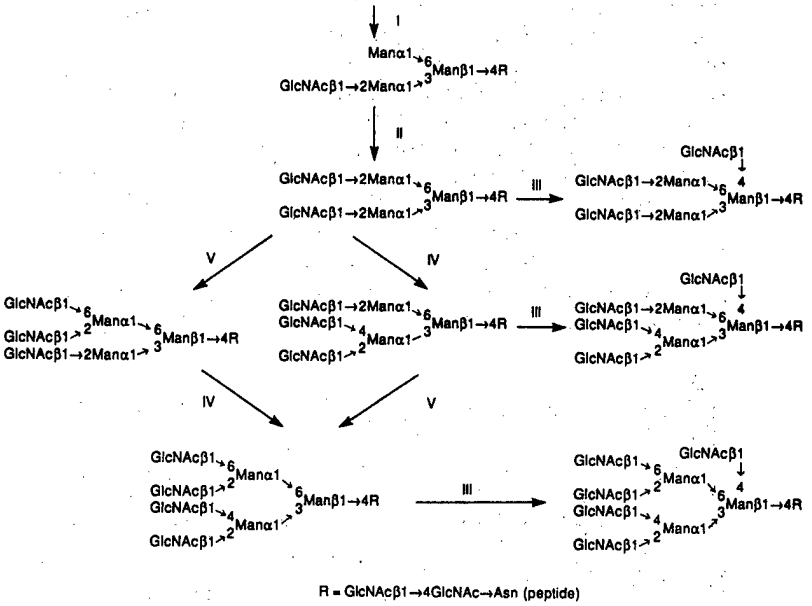

Fig. 2. Biosynthesis of a series of complex type sugar chains by the actions of GnT-II ~ V.

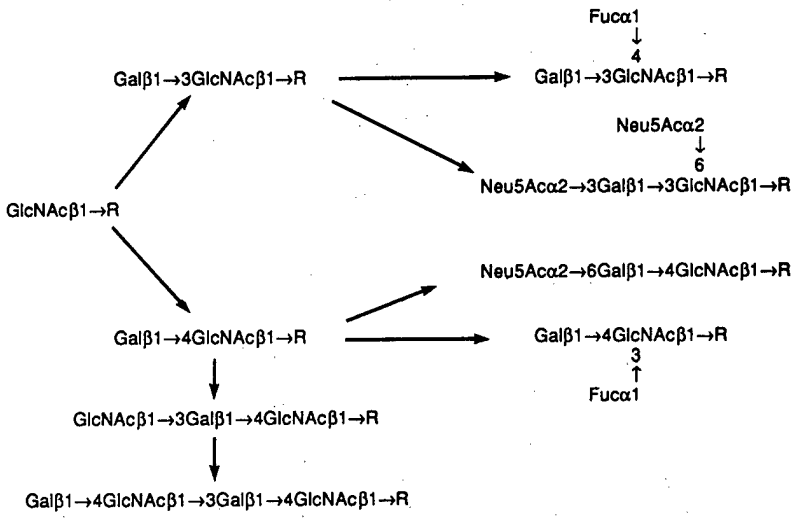

Fig. 3. Formation of various outer chains R represents the trimannosyl cores.

Similarly, prior sialylation prevents the following fucosylation by the α1,3-fucosyltransferase. Therefore, the ratio of sialylated and fucosylated outer chains must be determined by the ratio of the sialyl- and the fucosyltransferase activities expressed in a cell.

III. HOST CELL DEPENDENT PROTEIN GLYCOSYLATION —— SPECIES AND ORGAN SPECIFIC SUGAR CHAIN FORMATION.

The enzymes responsible for the processing of *N*-linked sugar chain are included in all cells. However, expression of the glycosyltransferases related to maturation of the sugar chain is quite different by animal species and by organs. The most clear-cut evidence for the species-specific and organ-specific glycosylation was provided by the systematic investigation of the sugar chains of γ-glutamyltranspeptidase (γ-GTP)[6]. This enzyme widely occurs in all mammals as a membrane integrated glycoprotein of epithelial cells of various organs. Only *N*-linked sugar chains are found in these enzymes. In Fig. 4, structures of major sugar chains of γ-GTPs purified from the kidneys and livers of various mammals are summarized.

Both kidney and liver enzymes of mouse contain biantennary sugar chains. However, the structures of outer chains of the two enzymes are totally different. Furthermore, the sugar chain of the kidney enzyme contain bisecting GlcNAc and core fucose, which are not found in the sugar chain of the liver enzyme. Therefore, organ-specific difference exists in the sugar chain of mouse γ-GTPs. Structural difference is also found in the sugar chains of kidney and liver enzymes of other mammals. Species-dependent glycosylation was also found to occur in both kidney and liver enzymes.

Presence of the species- and organ-specific difference in the glycosylation of proteins will afford many problems in the production of glycoproteins by recombinant technique. Actually, comparative study of the *N*-linked sugar chains of natural human interferon-β1 (IFN-β1) and three recombinant IFN-β1s produced by different mammalian cell lines transfected with the gene coding human IFN-β1 revealed that they all

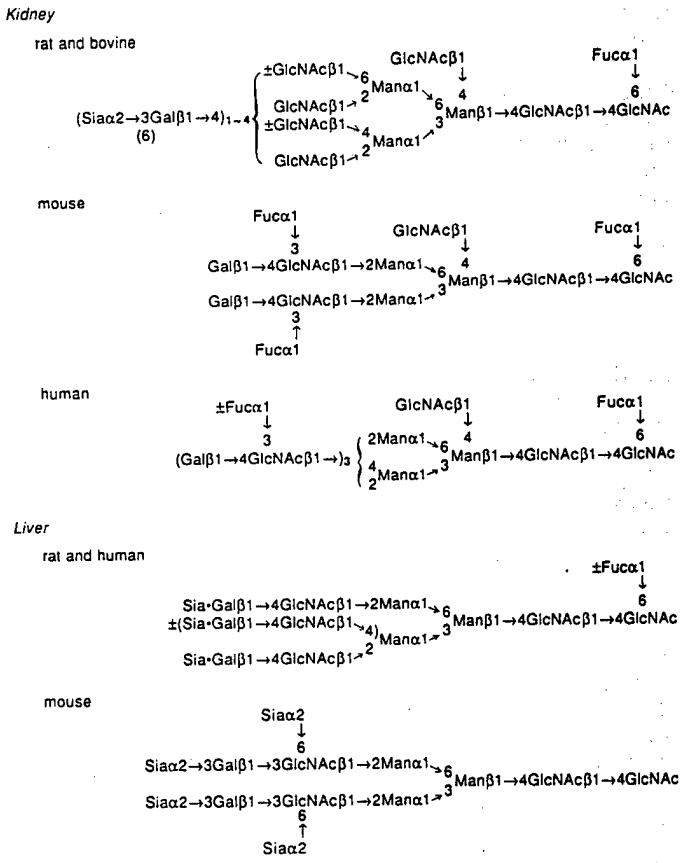

Fig. 4. Major sugar chain structures of γ-GTPs from the kidney and the liver of various mammals.

contain different sets of *N*-linked sugar chains[7]. More than 80% of the sugar chains of natural IFN-β1 had a biantennary structure. The remainder was composed of 2,4-branched (10%) and 2,6-branched (8%) triantennary sugar chains. Recombinant IFN-β1 produced by Chinese hamster ovary (CHO) cells contained sugar chains most similar to those of the natural counterpart. However, it contained increased amount of 2,6-branched triantennary sugar chains (31%) with a slight decrease of biantennary sugar chain and complete absence of 2,4-branched triantennary sugar chains. In addition to the increase of 2,6-branched triantennary sugar chains, appearance of a small amount of tetraantennary sugar chains (4 to 5%) was found in other recombinant IFN-β1s produced by mouse epithelial cells derived from breast carcinoma (C127) and by human lung adenocarcinoma cells (PC8). 2,4-Branched triantennary sugar chains did not occur in PC8 cell-derived IFN-β1 as in the case of CHO cell-derived IFN-β1. Host cell-dependent differences in glycosylation were observed not only in the antennary structures, but in the structures of outer chains and trimannosyl core. Natural and CHO cell-derived IFN-β1s were quite similar in that all of the sugar chains are not bisected and contain only the Siaα2→3Gal group. In contrast, sialic acids in the sugar chains of C127 cell-derived IFN-β1 exclusively occur as the Siaα2→6Gal group, and the Galα1→3Gal group was extensively expressed. The most remarkable characteristic found in the sugar chains of PC8 cell-derived IFN-β1 is the presence of bisected trimannosyl core, which is expressed in one fifth of the sugar chains. Organ-specific and species-specific expression of glycosyltransferases should be the enzymatic basis of the different sugar patterns of these recombinant IFN-β1s. Another factor, transformational change in the expression of glycosyltransferases must also be considered as the background of this phenomenon, because all host cell lines used for the production of recombinant IFN-β1s are tumor cells. The phenomenon of altered glycosylation of proteins is widely found in various tumors[8-10].

IV. EFFECT OF POLYPEPTIDE PORTION ON THE STRUCTURE OF MATURATED SUGAR CHAIN.

As described above, it is obvious that a key factor to determine the structures of sugar chains of a glycoprotein is the level and the set of glycosyltransferases expressed in the cell producing the glycoprotein. However, there are many examples indicating that distinct sites of a glycoprotein are differently glycosylated.
Human chorionic gonadotropin (hCG) is composed of α and β subunits, both of which have two *N*-linked sugar chains. The β subunit contains 1 mol each of biantennary sugar chains with fucosylated and non-fucosylated trimannosyl cores. In contrast, the α subunit contains 1 mol each of mono- and biantennary sugar chains with non-fucosylated trimannosyl core[11]. Since both subunits are synthesized by the same trophoblast, this evidence indicates that the conformation of each subunit control the maturation of the sugar chains attached to it. The phenomenon of protein-dependent glycosylation is more precisely documented by the fact that structural difference is observed among the different recombinant glycoproteins produced by the same host cells. For example, almost all of the sugar chains of recombinant interferon-γ produced by CHO cells are of biantennary complex type[12], while more than 80% of the sugar chains of recombinant erythropoietin produced by the same cells are of tetraantennary complex type[13]. As described already, recombinant human IFN-β1 produced by CHO cells contains bi- and triantennary sugar chains in a molar ratio of 7 to 37. This protein controlled glycosylation may be useful in making glycoproteins by recombinant techniques.

V. STUDY OF FUNCTIONAL ROLE OF THE SUGAR CHAINS WITH USE OF RECOMBINANT GLYCOPROTEINS.

As already discussed, many recombinant glycoproteins contain different sets of sugar chains from their natural counterparts. By investigating the biological activities and the sugar chain structures of these recombinant glycoproteins, a new approach to elucidate the function of the sugar chains of glycoproteins has been opened. As an example, recent studies on recombinant human erythropoietin (EPO) will be introduced below.

EPO is a haemopoietic hormone specific to cells in erythroid lineage. Human EPO consists of 165 or 166 amino acids, and contains three *N*-linked sugar chains and one *O*-linked sugar chain. Total sugar chains amount to 40% of the molecular weight of this glycohormone. Although desialylated EPO was more active than sialylated one as measured by the bioassay system *in vitro*, it showed no *in vivo* hormonal activity. Hence the functional role of the sugar chains of EPO had been attracting researchers attention. Successful cloning of the structural gene of human EPO in 1985 opened a way to obtain large amount of recombinant EPO. Takeuchi *et al.*[13] analyzed the structures of the *N*-linked sugar chains of recombinant EPO produced by CHO cells and of natural EPO purified from the urine of patients with aplastic anaemia. As summarized in

Table I. Structures of the *N*-linked sugar chains and their molar ratio of natural and recombinant EPOs. R=GlcNAcβ1→4(Fucα1→6)-GlcNAc$_{OT}$; R'=GlcNAcβ1→4GlcNAc$_{OT}$. *Natural EPO has α2→3 and α2→6 linkages. **The locations of the *N*-acetyllactosamine repeat in the sugar chains of natural EPO were not determined.

Structures	Percent molar ratio			
	rHuEPO		Urinary HuEPO	
	R	R'	R	R'
(NeuAcα2→3)$_{1-2}$ Galβ1→4GlcNAcβ1→2Manα1↘6 / Galβ1→4GlcNAcβ1→2Manα1↗3 Manα1→4R/R'	4.1	1.9	5.3	3.7
(NeuAcα2→3)$_{1-3}$ Galβ1→4GlcNAcβ1→2Manα1↘6 / Galβ1→4GlcNAcβ1↘4 Manα1↗3 / Galβ1→4GlcNAcβ1↗2 Manα1→4R/R'	4.8	4.1	2.4	1.2
(NeuAcα2→3)$_{1-3}$ Galβ1→4GlcNAcβ1↘6 / Galβ1→4GlcNAcβ1↗2 Manα1↘6 / Galβ1→4GlcNAcβ1→2Manα1↗3 Manβ1→4R/R'	2.4	2.1	15.1	4.9
(NeuAcα2→3)$_{1-4}$ Galβ1→4GlcNAcβ1↘6 / Galβ1→4GlcNAcβ1↗2 Manα1↘6 / Galβ1→4GlcNAcβ1↘4 Manα1↗3 / Galβ1→4GlcNAcβ1↗2 Manβ1→4R/R'	39.6	6.4	51.9	8.0
(NeuAcα2→3)$_{1-3}$ Galβ1→4GlcNAcβ1→3** Galβ1→4GlcNAcβ1↘6 / Galβ1→4GlcNAcβ1↗2 Manα1↘6 / Galβ1→4GlcNAcβ1↘4 Manα1↗3 / Galβ1→4GlcNAcβ1↗2 Manβ1→4R	30.2	0	6.9	0
(NeuAcα2→3)$_{1-2}$ Galβ1→4GlcNAcβ1→3** Galβ1→4GlcNAcβ1↘6 / Galβ1→4GlcNAcβ1↗2 Manα1↘6 / Galβ1→4GlcNAcβ1↘4 Manα1↗3 / Galβ1→4GlcNAcβ1↗2 Manβ1→4R	4.3	0	0.6	0

Table I, no qualitative difference was detected in the sugar chains of the two EPO samples, except for that the sugar chains of the recombinant EPO contains only the Neu5Acα2→3Gal group while natural EPO contains both the Neu5Acα2→3Gal and the Neu5Acα2→6Gal groups. This structural similarity warrants the usefulness of the recombinant EPO as a drug to treat anaemia.

During the course of studies on the effective production of EPO in recombinant CHO cell lines, a cell line, B8-300 was found to produce an EPO preparation with extremely low *in vivo* hormonal activity. Study of its sugar chains revealed that the EPO is enriched in biantennary sugar chains[14]. Comparative study of the sugar patterns and the *in vivo* activities of several preparations of recombinant EPO revealed that the activity was proportional to the ratio of tetraantennary and biantennary sugar chains. The low *in vivo* activity of the EPO obtained from B8-300 was not due to a defect in the activation of the biological response at the receptor level, since its *in vitro* activity was considerably higher than that of normal EPO[14].

Study of the effect of enzymatic trimming of the sugar chains on the *in vitro* activity of normal EPO revealed that removal of the outer chain moieties of its *N*-linked sugar chains increases the *in vitro* activity of EPO. The activity was gradually diminished by further trimming of the trimannosyl core portions, and finally lost by removing all *N*-linked sugar chains[15]. These results indicated that highly branched and sialylated *N*-linked sugar chains take part in targeting EPO to bone marrow, and the core portion of the sugar chains is important for the action of EPO on the target cells.

CONCLUDING REMARKS

Because of the limited amount of space, EPO was chosen as a topic of the functional study of the sugar chains of glycoproteins. Another recent report of interest is the study on the sugar chains of recombinant tissue plasminogen activator (TPA). An important evidence is that TPA is a mixture of two glycoproteins with two and three *N*-linked sugar chains, and the kinetics of the fibrinolytic activity of these two glycoproteins are quite different. Rademacher *et al.*[16] suggested that a mixture of the two glycoproteins in an appropriate ratio is necessary to dissolve thrombi effectively while suppressing the ill effect of TPA: acceleration of systemic fibrinolysis. This may indicate the complex nature of glycoprotein drugs.

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A-3-2

Testing Human Oncogenes in Transgenic Mice

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It is strongly suggested that the cancer development is essentially caused by the activation of oncogenes and/or inactivation of antioncogenes that were not only from germline mutations but also from somatic mutations

Many genes have been reported to be involved in carcinogenesis. *ras* family genes (Ha- Ki- and N-*ras*) are one of the genes that are reported to be involved in the regulation of cell proliferation and differentiation in normal eukaryotic cells (1). Moreover, activated types of *ras* oncogenes are frequently found both in human cancers and in chemically induced animal cancers. It appears to be implicated in tumorigenesis (1). A somatic point mutation in the 12th or 61st codon and less frequently in the 13th or 59th codon of the *ras* gene seems to acquire transforming activity in *in vitro* cell culture systems such as the NIH3T3 cell line. However, the *in vivo* effects of these mutational changes of the *ras* oncogens in the tissue-specific development of animal and of human cancers remain to be elucidated.

Animal model systems have been used to study the causative role of the *ras* gene in development of tumors and their tissue-specific activity *in vivo*. A many animal tumors in which *ras* oncogenes have been reproducibly found to be activated were induced by a variety of chemical carcinogens. For example, in rat mammary carcinomas induced by a single dose of N-methyl-N'-nitrosourea (MNU) during puberty, the c-Ha-*ras* oncogene was activated in 82% of the treated rats. The skin papillomas and carcinomas induced by the initiator dimethylbenzanthracene (DMBA) followed by multiple applications of the promotor 12-O-tetradecanoyl-phorbol-13-acetate (TPA) showed a very high frequency (90%) of activation of the c-Ha-*ras* oncogene in the mouse. These models also provide good systems for studying the mechanisms of the point mutational activation of *ras* genes.

Transgenic animal models that carry an activated *ras* gene under the control of a certain type of promoters and accomplish their targeted expression in the expected tissues were also generated. We have also generated unique transgenic mouse lines (rasH2, rasH7 and rasH8) with the proto-type human c-

Ha-*ras* gene with its own promoter. All of these lines developed tumors spontaneously; they were angiosarcomas, skin papillomas, lung and Harderian gland adenocarcinomas (2). Interestingly, the somatic mutations of the transgene were observed in these tumors, but not in the normal tissues (3). The type of the mutations was also tumor-specific; for instance, a base substitution of the codon 12 and 61 of the transgene was always detected in skin papillomas and angiosarcomas, respectively. These results suggested that the definite somatic mutation of the transgene in certain cell types was a causative event in tumorigenesis in these transgenic mice. However, with this approach the tumor incidence was about 50% and the latent period was as long as 18 months, since the mutational events were sporadic. Therefore, we administered alkylating agents, MNU and DMBA, to rasH2 transgenic mice in order to induce tumors more frequently as well as within a shorter latent period of time than in the natural conditions. MNU is known to cause the GC to AT transition very frequently at the codon 12 of the c-Ha-*ras* gene from GGC to GAC. On the other hand, DMBA is an agent which is known to cause the AT to TA transversion also very frequently at the codon 61 of the c-Ha-*ras* gene from CAG to CTG.

Transgenic mice were confirmed to be very susceptible to the chemical carcinogens, both of MNU and DMBA. Within 12 weeks after administration of MNU or DMBA, the transgenic mice developed forestomach papillomas and then carcinomas very frequently, at the rate of almost 1oo% and almost all of the tumors had point mutations at the 12th codon from GGC (Gly) to GAC (Asp) by MNU and the 61st codon of the transgenes from CAG (Gln) to CTG (Leu) by DMBA. Because any somatic point mutations in the transgene have never been detected in normal tissues of the transgenic mice in this short period, these mutations seemingly activated the human proto-type c-Ha-*ras* transgene.

Next, we tried to use these transgenic mouse lines for testing the chemical substance, azatyrosine, which is known to convert the NIH3T3 cells transformed by the activated *ras* genes to normal cell types. MNU was administered once intraperito-neally into transgenic mice, and then azatyrosine was introduced every other days for 12 weeks. All transgenic mice which were administered only with MNU, but not with azatyrosine, developed forestomach papillomas. However, all transgenic mice which were administered with MNU and then treated with azatyrosine have never developed forestomach papillomas at all (4).

From these results, it is suggested that the somatic mutation of the human c-Ha-*ras* transgene plays a causative role in the occurrence of the forestomach and skin papillomas induced by MNU or DMBA administration in transgenic mice. This transgenic mouse provides a unique screening system for chemicals that induce or suppress the tumorigenesis.

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A-3-4

MULTIPLE GENETIC ALTERNATIONS IN HUMAN CARCINOGENESIS

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ABSTRACT

Multiple steps are required for conversion of a normal cell to fully malignant cancer cells with metastatic and invasive capacity. During the multi-step carcinogenic process, multiple genetic changes occur involving those of proto-oncogenes and tumor suppressor genes. We have focused studies on genetic changes in the late stage of carcinogenesis. We have shown here amplification of newly isolated gene, *K-SAM*, in poorly differentiated type of stomach cancer but not in differentiated type. Frequent amplification of 11q13 regions containing *HST1*, *INT2*, cyclin D/*EXP2* and *EXP1* gene in esophageal cancer was also shown together with multiple amplification units in a cancer cell. Our results of studies on scenarios of multiple genetic changes in various cancer are also reviewed and discussed together with those reported from other laboratories.

INTRODUCTION

To convert a normal cell to fully malignant cells with invasive and metastatic capacities, several carcinogenesis steps are required, involving steps for initiation, promotion and progression, which usually require 10 to 20 years. For each carcinogenic step, a genetic change occurs, including activation of proto-oncogenes and inactivation of tumor suppressor genes. Infection of virus can also be considered to be a genetic alternation. We have been studying genetic changes in various human cancers, and some of scenarios of genetic changes specific to each type of cancer have been elucidated. We have also been studying gene amplification, which occurs in the late stage of carcinogenesis and probably contributes malignant phenotype of cancer. The results of our studies on multiple genetic changes in cancer will be presented together with those reported from the other laboratories, and significance of genetic changes, especially that of gene amplification in diagnosis and treatment of cancer will be discussed.

RESULT & DISCUSSIONS

One of the characteristics of a cancer cell is aneuploidy and increased content of DNA is often associated with increased malignancy of cancer. More than 6 proto-oncogenes have been identified and characterized, and most of the products of these genes are considered to play crucial roles in signal transduction pathway. There have, however, been only limited number of proto-oncogene reported to be altered in human cancer. *MYC* family, *RAS* family, c-*ERB*B-1 and c-*ERB*B-2 are the proto-oncogenes reported to be amplified in some types of cancers. It is very likely that there are yet-to-be-discovered genes which plays important roles in development of human cancer. Accordingly, we decided to search for amplified genes which gave growth advantage to cancer cells in the late stage of carcinogenesis.
In-gel DNA renaturation method was initially used to detect amplified genes in human cancer[1]. Cancer we have studied to identify amplified genes was KATOIII cells, an established cultured cell line derived from schirrus type of stomach cancer[2]. Stomach cancer is still the most common cause of cancer death in Japan, and one of the most common cancer in the world. Yet, there have been little information available for genetic changes in stomach cancer. By in-gel DNA renaturation method, we identified many amplified DNA sequence. One of the amplified sequence was cloned directly from the gel, and by genomic walking and cDNA cloning, a new amplified gene was identified. The gene was designated as K-*SAM*, and the nucleotide sequence analysis of cDNA of K-*SAM* predicted that K-*SAM* encoded a transmembrane type of receptor with tyrosine-kinase activity. We have isolated cDNA for K-*SAM* related gene, N-*SAM*, and N-*SAM* was turned out to be quite homologous to chicken bFGF receptor and later found to be identical to *FLG* or FGFR1. Thus, K-*SAM* was considered to encode a receptor for one of a FGF family growth factor. K-*SAM* was later found to be identical to *BEK* or FGFR2. K-*SAM* was amplified in 20% of poorly differentiated type or diffuse type of stomach cancer, but not in well differentiated type or intestinal type. In contrast, c-*ERB*B-2 was amplified in 40% of well differentiated type of stomach cancer but not in poorly differentiated type. Immunohistochemical analysis showed that K-*SAM* product was highly expressed in metastatic lesions of poorly differentiated type of stomach cancer. It was also shown that there were at least 4 types of K-*SAM* mRNAs generated possibly by alternative splicing, and presence of soluble K-*SAM* products without transmembrane domains were predicted[3]. Ligand(s) for K-*SAM* proteins is a growth factor belonging to FGF family or heparin binding growth factor family. It includes aFGF, bFGF, *INT2* protein, *HST1* protein. FGF5, *HST2*/FGF6 and KGF. It was reported from other laboratory that the ligand for K-*SAM* could be KGF.
We have previously isolated *HST1* gene as a transforming gene in a stomach cancer using NIH3T3 transfection assay[4]. Unlike aFGF and bFGF, *HST1* protein contains a signal peptide and excreted outside of the cells. The *HST1* protein was shown to have a potent angiogenic activity and transforming activity. *HST1* was localized on human chromosome 11q13 about 35kb apart from *INT2*[5]. The *HST1* and *INT2* was amplified in 40% of esophageal cancer and 20% of breast cancer. It was found that 100% of lymph node metastasis of esophageal cancer contained amplified *HST1* gene.
There has, however, been no detectable mRNAs for *HST1* or *INT2* in esophageal cancers, even though these cells contained amplification of the genes. It was likely that there were other unknown genes, near

HST1 and *INT2* which was amplified and expressed frequently in esophageal cancer and gave growth advantage for these cancer cells. By cosmid walking, two genes were identified on the same amplicon containing *HST1-INT2* genes on 11q13. One is designated as *EXP1*. and the other is *EXP2*. Sequence analysis of corresponding cDNAs showed that *EXP2* was identical to recently reported cyclin D gene, while the product of *EXP1* gene remains unknown. We had recently identified the other gene on this amplicon on 11q13 by an exon trapping. These results showed at the first time that one amplicon contains at least five genes one of which was considered to provide esophageal cancer cells with growth advantages. In addition, we have recently found presence of quite a few amplicons in a single cancer cell and different amplicons were isolated by a genomic subtraction method.

We have reported in 1984 presence of K-*RAS* point mutation, amplification of a point mutated K-*RAS* and c-*MYC* in a pancreatic cancer, providing us with a case containing multiple genetic changes[6]. The cancer cells later were shown to contain mutation of p53 gene. It was reported that K-*RAS* point mutation occurred in 80% of pancreatic cancer. K-*RAS* point mutation, however, seldom observed in stomach cancer and small cell lung cancer, indicating that type of gene involved for alteration were depended upon the types of tumors. Based on the experimental results, we have initiated to study genetic changes in several cancers.

In small cell lung cancer, loss of heterozygosity (LOH) on 3p, 13q and 17p occurred in 100% of the cases, while LOH on 3p was observed in 80% of adenocarcinoma of lung. In some cases, amplification of *MYC* proto-oncogene was detected[7]. LOH on 13q and 17p were later found to involve *RB* gene and p53 gene, respectively. The remaining allele of *RB* gene or p53 gene in small cell lung cancer was shown to have mutation. In stomach cancer, p53 gene mutation was frequently found. In differentiated type of stomach cancer, *RAS* point mutation and c-*ERB*B-2 amplification could be detected in some cases. Poorly differentiated type contains no *RAS* mutation and c-*ERB*B-2 amplification but some had K-*SAM* amplification. In cervical cancer, LOH at 3P was quite frequently found in addition to HPV infection[8]. It was shown that E7 and E6 protein of HPV type 16 bound to *RB* protein and p53 protein, respectively and inactivated these tumor suppressor gene products at the protein levels. The p53 gene is the gene most commonly reported to be altered in human cancers. The p53 gave mutation was found in esophageal cancer, colon cancer, liver cancer, ovarian cancer, endometrial cancer and bladder cancer of invasive type but not in superficial type. The mutation of P53 gene as well as c-*ERB*B-2 amplification were good markers for poor 5-year survival for breast cancer. Scenarios of multiple genetic changes in colon cancer were clearly shown by the data from other laboratories, involving *APC* or *MCC*, *RAS*, p53 gene and *DCC*. The presence of a scenarios of multiple genetic changes each specific to origin of tissues or a cell from which cancer develops is now well established, and presence of multiple genetic changes in the clinical cancer has been well documented. Significance of these multiple genetic changes will be discussed in relationship to prevention, diagnosis and treatment of cancer.

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APPENDIX

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A-4-4

ISOLATION AND CHARACTERIZATION OF ESTROGEN RESPONSIVE GENES FROM HUMAN GENOME

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ABSTRACT

Estrogen not only plays important roles in feminization of various reproductive organs but also appears to play crucial roles in extragonadal development including central nervous system. Although most estrogen actions are mediated by estrogen receptor (ER) that binds to estrogen responsive genes, relatively few of which are identified so far. In order to search for those genes, we have developed a novel procedure in which human genomic DNA fragments were selected for the presence of the estrogen response element (ERE) by binding to the E. coli-expressed DNA-binding domain of the rat ER. Repetition of this type of screening yielded several clones having the authentic palindromic ERE, some of which showed transcription enhancing activities when ligated to the standard promoter-CAT reporter construct. By using genomic contiguous fragments as probes for Northern-and Zoo-blots, we have identified a gene that is expressed in human placenta. The gene encodes a novel protein having multiple Zn-finger domains.

INTRODUCTION

Although a wide variety of estrogen action are described on different organs and tissues, relatively few genes are known that are responsive to estrogen receptor (ER). Estrogen responsive genes that have been identified so far include those of vitellogenin (1), prolactin (2), pS2 (3), ovalbumin (4) and progesterone receptor (5). More important genes which should regulate the growth of female organs such as mammary gland and uterus in response to estrogen, for example, have not yet been found. Moreover, ERs have also been identified in various nuclei of female as well as male central nervous system (6-9) and in bone cells (10-11) implicating some important roles of estrogen responsive genes in a number of extragonadal organs. Since identification of more estrogen responsive genes appears crucial for more understanding of the molecular physiology of estrogen action, we have attempted to screen and isolate estrogen responsive genes by a novel procedure.

The rationale is that the binding specificity of the DNA-binding domain of the ER (ER-DBD) could be utilized to isolate the DNA elements in genomic DNA that flank the estrogen responsive genes. Therefore, we produced a protein of the ER-DBD in *E. coli* and screened human genomic fragments by selection cycles with filter binding. Five ER-binding fragments containing perfect palindromic estrogen responsive element (ERE) sequences were isolated and some of these fragments showed estrogen-dependent enhancer activities (12). Furthermore, we identified a protein-coding region adjacent to one of these sequences.

MATERIALS AND METHODS

Experimentals have been described in a previous paper (12) in detail. Other procedures will be published elsewhere.

RESULTS

The production and purification of the ER-DBD protein in *E.coli*

A DNA fragment containing the whole ER-DBD was expressed in *E. coli.* The cells were disrupted by sonication and the inclusion bodies accumulated were collected from the sonicated lysate by sucrose gradient centrifugation. They were denatured and solubilized in 8 M urea and renatured by step dialysis in the presence of 0.05 mM ZnCl2. SDS-polyacrylamide gel electrophoresis showed that the recombinant protein was purified to near homogeneity (Fig.1a). The protein migrates with a Mr of 14 kDa which corresponds to the caluculated Mr (14,039 Da) of the ER-DBD protein.

The binding specificity of the ER-DBD protein

We confirmed the DNA-binding specificity of the ER-DBD protein by filter binding assay and footprinting. The vitellogenin A2 enhancer sequence containing an ERE was cloned into plasmid pUC18. The plasmid (pUC/vitERE0 was digested with HpaII and EcoRI and 3'-end labeled with

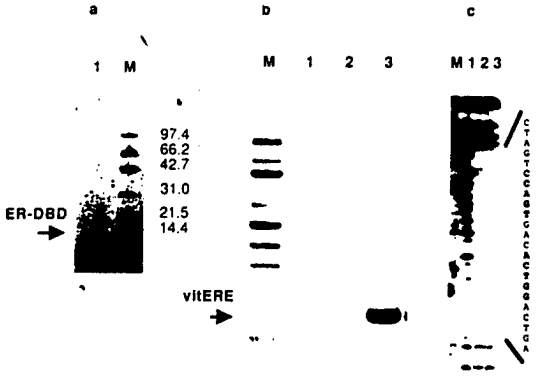

Fig.1. The purity and binding specificity of the ER-DBD protein. (a) SDS-gel with markers (M). (b) Filter-bound proteins with 0 (lane 1), 0.2 (lane 2) and 5 (lane 3) pmol of ER-DBD protein. M represents the total HpaI/EcoRI fragments of pUC/vit ERE.(c) Footprints of vitERE by 0 (lane 1), 1 (lane 2) and 2 (lane 3)pmol of ER-DBD protein. M is a sequence ladder A (> C).

[α -^{32}P]dCTP. The labeled fragments were mixed with the ER-DBD protein and bound fragments were trapped by nitrocellulose filter. Only the fragment containing the ERE of vitellogenin enhancer was trapped (Fig.1b: lane 2,3),. Other fragments derived from plasmid pUC18 sequence failed to bind the protein and passed through the filter. The sequence protected by the ER-DBD protein was then determined by DNase I footprinting. The single-end labeled DNA fragment containing the ERE of vitellogenin A2 enhancer was prepared from the pUC/vitERE plasmid, incubated with the ER-DBD protein and then digested with DNase I. The protected sequence was the consensus sequence of ERE (Fig.1c: lane 2, 3) and corresponded to the binding sequence of the intact ER (39).

Isolation of the ER-binding fragments in genomic DNA

The fragments bound to the ER-DBD protein were selected by nitrocellulose filter. The selection process shown in Fig.2 was repeated six times and five independent clones were isolated. These plasmids containing inserts ranging from 200 bp to 2 kb were named E1-E5. Sequence analysis has shown that all of these fragments contain perfect palindromic ERE sequences termed here ERE1-ERE5, respectively (Fig.3). Southern blot analysis for the E2 and E3 fragments showed that these fragments were present in single copy or at most a few copies in human genome (data not shown). In addition to the E1-E5 fragments, we also isolated seven ER-binding fragments from genomic DNA digested with Sau3AI by the whole genome PCR method (13). Only one of them contained the perfect ERE sequence and was named Epcr. Interestingly, the Epcr was a part of the E3 fragment as demonstrated by the presence

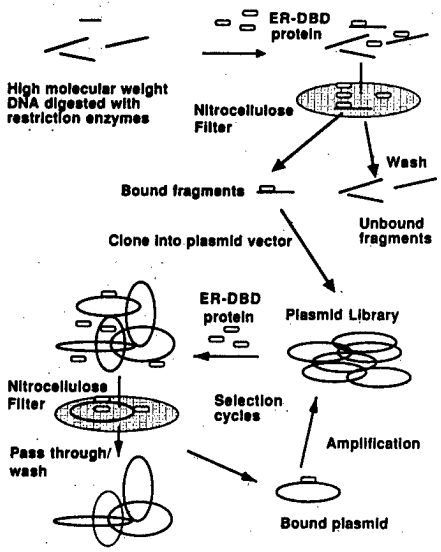

Fig.2.The strategy for the screening of ER-binding fragments in genomic DNA.

of identical ERE3 and neighboring sequences (data not shown). These findings indicate that we have isolated the ER-binding fragment derived from the same locus in the genome by two independent experiments with similar but slightly different procedure.

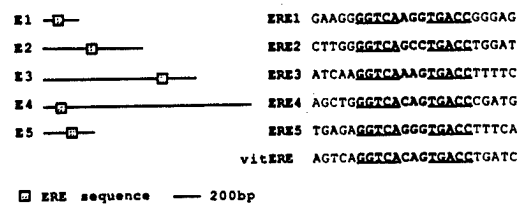

Fig.3. The ER-binding fragments and their ERE sequences.

Enhancer activity of the ER-binding fragments

We wanted to know if these ER-binding fragments have any estrogen-dependent enhancer activity. The whole ER-binding fragments, E1-E3, were inserted into a reporter vector pBLCAT2 having a Herpes simplex virus tk promoter to construct the E1-tk-cat, E2-tk-cat, and E3-tk-cat, respectively. The reporter plasmids were co-transfected with the ER expression vector pSV2RcER into COS-7 cells. As controls, the reporter plasmids without insert (tk-cat) and with the ERE of vitellogenin enhancer (vitERE-tk-cat) were assayed simultaneously. 12 hr after transfection, the cells were divided into two dishes and cultured in the presence or absence of estradiol. After 24 hr of culture, the CAT activities were assayed (Fig.4). The ER expression vector was able to stimulate vitERE-tk-cat in the presence of estradiol but not tk-cat itself. The estrogen-dependent enhancer activity was demonstrated clearly for the E1-tk-cat, although some background activity was noted even without the ligand. For E2-tk-cat and E3-tk-cat, the enhancer activity was undetectable. To examine the possibility that the surrounding sequences may interfere with the enhancer actibity, the short sequences containing

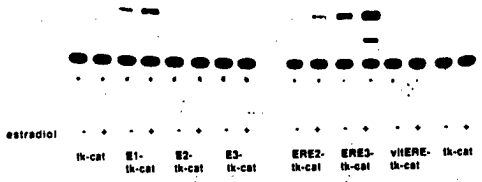

Fig.4. The estrogen-dependent enhancer activity of the ER-binding fragments and their EREs. The reporter plasmids containing E1-tk-CAT, E2-tk-cat, E3-tk-cat, ERE2-tk-cat, ERE3-tk-cat, vitERE-tk-cat and tk-cat were transfected into COS7 cells with the ER expression vector. After culturing in the absence or presence of $1x10^{-7}$M 17β-estradiol, CAT assay was performed.

the ERE2 and ERE3 which are the perfect palindromic ERE in the E2 and E3 fragments were cloned into the reporter vector pBLCAT2 (ERE2-tk-cat and ERE3-tk-cat) and tested for the enhancer activity. These minimum ERE sequences indeed showed certain estrogen-dependent enhancer activities, suggesting that some surrounding sequences have an interfering or silencing activity in COS-7 cells.

Isolation of a novel estrogen responsive gene

We have further cloned human genomic fragments hybridizing with the DNA fragments described above and selected clones by the criteria of the presence in the Zoo blot and Northern blot to various organs. As a result, a cDNA clone of 2.5 kb was obtained from human placenta library. The sequence of the cDNA clone contained an open-reading frame encoding protein having three potential Zn-fingers (data not shown).

DISCUSSION

We have isolated in this work human genomic DNA fragments that specifically bind to ER-DBD protein. Only perfect palindromic ERE sequences were cloned suggesting their special affinity to the ER-DBD protein. The Xenopus vitellogenin gene A2 estrogen-inducible enhancer has this sequence to which the ER expressed in HeLa cells binds tightly in the presence of estrogen (14). In this study, we have established that the sequences containing perfect palindromic ERE with 3bp spacing exist in human genome and also the DNA-binding domain of the estrogen receptor alone could specifically recognized and bind to the consensus ERE (GGTCANNNTGACC) without other factors which might help binding (15). It has been reported that imperfect palindromic ERE or half-site palindromic EREs are active as the estrogen-dependent enhancer in vivo in such genes as rat prolactin (2), human pS2 (3), chicken ovalbumin gene (4) and Xenopus vitellogenin gene (B2 enhancer, 16,17). We could not isolate these sequences in this work. They may require other factor(s) to bind tightly to ER. Or, they did not have strong enough binding capacity to ER to survive repeated screening used in this study. By decreasing the stringency of binding or just by decreasing the number of recycling, we may be able to isolated more regulatory sequences that bind to ER. In any event, isolation and identification of an estrogen-responsive gene by this novel procedure would open up a new general approach to the sturdy of the genes that are regulated by different transacting factors, such as steroid-thyroid-retinoid receptors, oncogenic transactivators etc.

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PROTEIN SECRETORY SYSTEM OF *BACILLUS BREVIS* AND ITS APPLICATION

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ABSTRACT

We developed a host-vector system in which protein-hyperproducing *Bacillus brevis* strains are used as host bacteria and the 5' region of a cell wall protein gene is utilized to construct expression-secretion vectors. This system has been used to produce large amounts of various useful proteins. Many bacterial proteins such as amylases could be produced more than 1 g/l with this system. The amounts of animal proteins produced with our *B. brevis* system were approximately 100 fold less than those of bacterial proteins, except for human epidermal growth factor (about 1 g/l).
The use of appropriate signal peptides and hypersecreting host mutants, and suppression of extracellular protease action were important for improving the efficiency of the production of some human proteins. The optimization of cultural condition for each protein was necessary to increase the production yield.

INTRODUCTION

As a result of extensive screening, about 20 strains of *Bacillus brevis* were isolated from soil and others as efficient producers of extracellular proteins.[1, 2] Two of the strains, 47 and HPD31, used most often in our work, accumulates up to 30 g proteins per liter of medium under appropriate cultural conditions. Utilizing this protein-hyperproductivity of *B. brevis*, we developed a host-vector system for very efficient synthesis and secretion of foreign proteins.[3] Its low extracellular protease activity in addition to the very strong capability of protein production made *B. brevis* an attractive and promising host.

MECHANISM OF EXTRACELLULAR PROTEIN PRODUCTION AND CONSTRUCTION OF EXPRESSION VECTOR

The cell surface structures of the protein-producing *B. brevis* strains

were found to be different from those of many Gram-positive bacteria. *B. brevis* 47 has a three layered cell wall consisting of two protein layers and a thin peptidoglycan layer. The two protein layers, termed the outer wall (OW) and middle wall (MW) layers, are composed of proteins with molecular weight of 103,000 (OWP) and 115,000 (MWP), respectively.[4] These cell wall proteins were indistinguishable from two major proteins secreted by *B. brevis* 47. The cell wall structure was morphologically altered depending on the growth period. At the early stationary phase of growth, cells began to shed the outer two surface layers. The morphological alterations in the cell wall occurred concomitantly with a prominant increase in protein secretion. The synthesis and sercretion of cell wall proteins continued even after cell growth ceased. They did not stay on the cell surface and accumulated in medium as extracellular proteins.[3]

The genes coding for the cell wall proteins were cloned and their nucleotide sequences determined. The two genes constitute an operon (cwp, cell wall protein operon). The 5' region of the cwp operon contains multiple and tandem promoters from which a very efficient transcription starts.[5] Both MWP and OWP were synthesized in precursor forms with their respective signal peptides. A 600 basepair *Alu*I-*Alu*I fragment containing all the multiple promoters, the translation initiation sites and the MWP signal peptide-coding region was isolated and used to construct expression and secretion vectors.[3] pNU210, one such vector with a multicopy number, had the *Alu*I-*Alu*I fragment, the erythromycin resistance gene, and the replication origin of pUB110. Another vector, pHY700 carries the replication origin of pWT481 found in *B. brevis* 481.[1] Although the copy number of pHY700 is low, it is maintained stably in *B. brevis* even in the absence of the selection drug. Foreign gene is inserted to the vector so that the DNA encoding the mature part of the secretory protein is fused directly to the carboxyl end of the MWP signal sequence. The plasmid thus constructed can be introduced to *B. brevis* by the Tris-PEG method or electroporation.[6,7]

PRODUCTION OF FOREIGN PROTEINS

Bacterial Proteins

Proteins of various bacterial origins, such as the thermophilic α-amylase of *Bacillus lichemiformis* and β-amylase of *Clostridium thermosulfurogenes*,[8] could be produced in large amounts with the *B. brevis* system, as shown in Table 1. It is noteworthy that the amount of β-amylase (1.6 g/l) of *C. thermosulfurogenes* secreted by *B. brevis* is about 60 times larger than that produced by a hyperproducing mutant of *C. thermosulfurogenes*.[8] Glucose isomerase of *Thermus thermophilus* was produced very efficiently in *B. brevis* (about 1.3 g/l), while *T. thermophilus* cells have only a small amount of the enzyme (about 1 mg/l).[9]

Table 1. Production of foreign proteins by *B. brevis*

Origin	Protein	Amount Produced (g/l)	Localization
Bacillus licheniformis	α-Amylase	3.5	Extracellular
B. stearothermophilus	α-Amylase	3.0	〃
B. macerans	Cyclodextrin glucanotransferase	1.0	〃
Clostridium thermosulfurogenes	β-Amylase	1.6	〃
Thermus thermophilus	Glucose isomerase	1.3	Intracellular
Human	Epidermal growth factor	1.1	Extracellular
	Interleukin 2	0.05	〃
	Salivary a-amylase	0.06	〃
Swine	Pepsinogen	0.01	〃
Aspergillus oryzae	Takaamylase A	0.02	〃

Animal proteins

The amounts of aminal proteins produced in our *B. brevis* host-vector system were much smaller than those of bacterial proteins, except for human epidermal growth factor which was secreted very efficiently (Table 1). The reasons for the differece in productivity between bacterial and animal proteins are now becoming apparent, so that the efficiency of animal protein production is improving to a greater extent.

Human epidermal growth factor (EGF)
EGF is a polypeptide composed of 53 amino acids with 3 intramolecular disulfide linkages. A synthetic EGF gene was insertd into the cleavage site of the MWP signal sequence on pNU200, so that the signal peptide was directly followed by mature EGF. *B. brevis* HPD31 carrying the plasmid produced as much as 0.24 g/l of hEGF under an appropriate cultural condition.[10] The EGF thus produced had the same amino-terminal amino acid sequence and biological activities as those of authentic EGF. pHY700EGF was constructed by inserting the EGF gene into pHY700 which is a stable plasmid described above (Fig. 1). As shown in Fig. 2, *B. brevis* harboring pHY700EGF produced 1.1 g/l of EGF.[11]

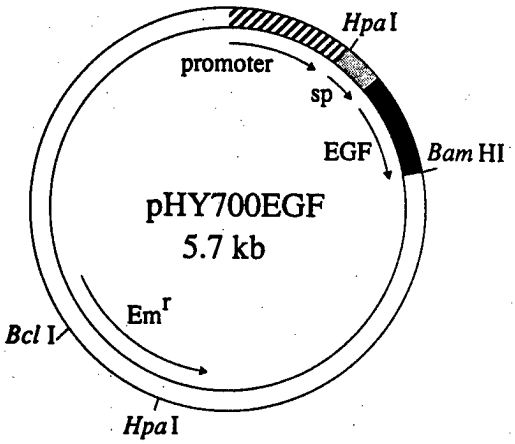

Fig. 1. Structure of Expression vector pHY700EGF. Promoter, the 5' region of the cell wall protein gene of *B. brevis*; sp, the signal sequence region of the cell wall protein gene of *B. brevis* 47; Em^r, erythromycin resistance gene.

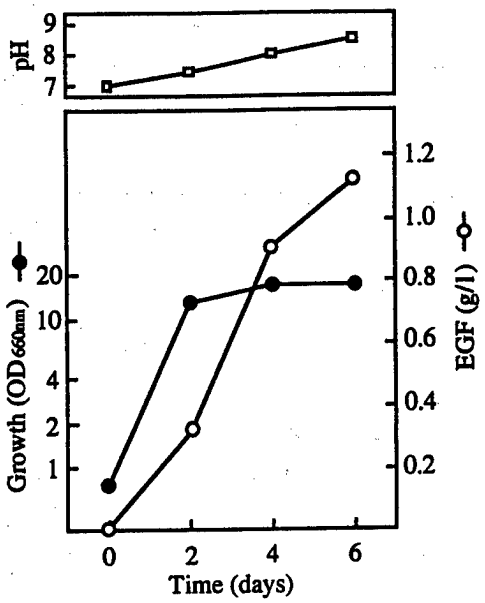

Fig. 2. Course of EGF production by *B. brevis*(pHY700EGF).

Human interleukin 2 (IL2)

IL2 is a medically important cytokine whose molecular weight is about 15000. It has one disulfide linkage and a cysteine residue. The gene encoding the mature IL2 was linked directly to the MWP signal sequence of pNU210. *B. brevis* carrying this plasmid secreted only a small amount of IL2 (about 1 mg/l). As a result of intensive studies, we found that IL2 was decomposed to fragments by protease(s) whose activity was hardly detected with the ordinary assay and large amounts of IL2 in precursor and mature forms were found associated with cells. The decomposition was prevented by the addition of protease inhibitor and mature IL2 was released into the culture supernatant when more hydrophobic signal peptide was used in place of MWP signal peptide. In this way, IL2 was efficiently produced by the *B. brevis* system (about 50 mg/l).

Human salivary α-amylase

Human salivary α-amylase consists of 511 amino acid residues including 11 Cys residues with a molecular weight of about 56,000. We constructed the direct fusion of the MWP signal peptide-coding sequence and the mature α-amylase-coding sequence. The fused gene was insertd into a low copy number vector plasmid (pHAMY5). Although B. brevis 47 (pHAMY5) secreted only a small amount (about 1 mg/l) of the enzyme, we were able to isolate a host mutant (B. brevis 47K) that secretes an increased amount of the enzy after mutagenesis with N-methyl-N'-nitro-N-Nitrosoguanidine. B. brevis 47K (pHAMY5) secreted 6 mg/l of the enzyme. Further increase in the enzyme production (60 mg/l) was attained when the MWP-α-amylase fused gene was inserted into a multi-copy vector pNU200 and introduced into *B. brevis* 47K[12]. The mutant showed higher sensitivity to various antibiotics than the parental strain, and alterd cell wall and cytoplasmic membrane protein compositions.

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B-1-5

GENETIC ENGINEERING FOR PRODUCTION OF MILK-CLOTTING ENZYMES

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ABSTRACT

Genetic engineering for production of two aspartic proteinases as milk coagulants in cheese industry is reviewed. Calf chymosin is produced through the processes, which consist of heterologous expression of prochymosin cDNA in E. coli and solubilization-refolding-processing of prochymosin peptide formed as inclusion bodies. A fungal gene encoding prepro-Mucor rennin is expressed in S. cerevisiae, which efficiently secretes the correctly processed active enzyme into the medium. These two expression systems are useful to conduct protein engineering of these enzymes, and allows to improve their practical properties such as the relative milk-clotting acitivity and heat-stability.

INTRODUCTION

Chymosin is an aspartic proteinase obtained from the stomach of suckling calves. It has a high milk-clotting activity along with a very low proteolytic activity. These properties make chymosin very suitable for the coagulation of milk, the first step in the processes of cheese manufacturing. On the other hand, shortage of chymosin in the market prompted to develop several substituting enzymes, among which fungal aspartic proteinases produced by two closely related species Mucor pusillus and M. miehei (Mucor rennin) are most successful.
Recombinant DNA techniques have opened new aspects in this field; (i) it allows to produce real chymosin irrespective of the fluctuating supply of its native resource, (ii) it allows to improve the slightly inferior properties of Mucor rennin by site-directed mutagenesis, and (iii) it allows to start systematic protein engineering to understand the catalytic mechanism in aspartic proteinases.

I. PRODUCTION OF CHYMOSIN IN *E. COLI*

Chymosin is secreted from the mucosal tissue of calf stomach as an inactive precursor, prochymosin, which in turn is autocatalytically processed to chymosin by cleaving the NH_2-terminal 42 amino acids under an acidic condition. We cloned prochymosin cDNA and constructed several expression plasmids to produce prochymosin polypeptide under the control of the *lac*- or *trp*-promoter in *Escherichia coli* (1-4). These plasmids encode fMet-prochymosin or fused prochymosins with a short NH_2-terminal exchange with Lac, TrpE or TrpL polypeptides, and directed production of these polypeptides in large amounts as inclusion bodies in *E. coli* cells. After solubilization of the inclusion bodies with 8M urea, fMet-prochymosin and most of the fused prochymosins were correctly refolded under highly alkaline conditions at pH 10-11 to a form which was activated by incubation at pH 2.3 (5). The only exception was a fused prochymosin CR601, in which the NH_2-terminal 4 amino acids of prochymosin were substituted with the NH_2-terminal 8 amino acids of the *trp*-leader peptide (6). Chromatographic analyses of the alkaline dialyzate of CR601 proved that the polypeptide was misfolded to various conformations which could not be activated by the acid treatment. These results indicate that a few amino acid residues at the region proximal to the NH_2-terminus of prochymosin play a crucial role in determining the course of *in vitro* refolding and final conformation of the whole polypeptide chain.

II. EXCRETION OF MUCOR RENNIN BY RECOMBINANT YEAST

The chromosomal gene of *M. pusillus* rennin (MPR) without intron sequences encodes for the pre-pro-enzyme containing an NH_2-terminal 66 amino acids extpension (7). The gene was expressed under the control of the *GAL7*-promoter in *Saccharomyces cerevisiae*. The correctly processed acitive enzyme was secreted to the medium at the concentration of more than 200 mg/l (8). A slightly larger protein cross-reacting with the anti-MPR antibody was also detected in the medium at the early stage of cultivation. Its NH_2-terminal amino acid was identified as the -44th Agr in the pre-pro sequence. Autocatalytic acitvation of the proMPR occurred at acidic pHs. These results indicate that the fungal signal sequence of prepro-MPR functions effectively for secretion of pro-MPR from yeast cells (9).
The mature MPR secreted by yeast had a distinctly higher molecular weight than the authentic MPR produced by the original fungus. On the other hand, the yeast MPR showed a lower ratio of the milk-clotting activity (C) to the proteolytic activity than did the original fungal enzyme. Treatment of the yeast MPR with endoglycosidase H caused a decrease in molecular size along with improvement of the C/P ratio. Hyper-mannosylation at the two N-glycosylation sites in the enzyme may affect its catalytic activity as well as substrate specificity through some perturbation effect on the enzyme structure. The glycosylation can be prevented by exchange of the two Asn in the glycosylation sites to Gln. Decreased secretion of the mutant MPR from yeast cells was improved by mutation of the host.

III. SITE-DIRECTED MUTAGENESIS OF CHMOSIN AND MUCOR RENNIN

Various mutant chymosins were generated by insertion of 2 or 4 amino acids by linker DNA insertion or single amino acid exchange by site directed mutagenesis and their catalytic properties were examined (10). Two amino acids (Ser-Arg) insertion between Pro107-Gly108 and single amino acid exchange of Tyr75 to Phe caused a significant increase in the C/P ratio. Kinetic parameters of the latter mutant Y75F were determined by using two synthetic peptides: Leu-Ser-Phe(NO_2)-Nle Ala-Leu-OMe(peptide I) and Lys-Pro-Ile-Glu-Phe(NO_2)-arg-Leu-OH(peptide II). A marked drop of k_{cat} but almost no change of K_m was observed with peptide I, while a marked increase in K_m but no change of k_{cat} was observed with peptide II.
Systematic exchange of the corresponding Tyr75 in MPR have revealed that (i) exchange with almost all amino acids except for Phe, (Trp) and Asn(!) caused total loss of the catalytic acitivity, (ii) Tyr75Phe showed almost the identical properties to those of the corresponding mutant of chymosin, and (iii) Tyr75Asn showed the improved properties in both the relative milk-clotting activity and the heat-stability.

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B-3-5

PERSPECTIVES IN MARINE BIOTECHNOLOGY

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What is Marine Biotechnology ?

Marine biotechnology encompasses the search, collection and improvement of ocean dwelling organisms, and the discovery and utilization of special functions and useful substances from microbes animals and plants from marine habitats. In order to make progress in marine biotechnology other supporting technologies are necessary, including the development of sensors, communications, and new bio-materials. The purpose of this paper is to review current Marine Biotechnology research strategies for the most efficient utilization of our marine resources. I hope to put our current research in a long term framework, with a final goal being the construction of a marine industrial complex capable of large scale total ocean utilization (Figure 1). The current level of global activity in the field of Marine Biotechnology indicates that this vision may be realized early in the 21st century.

Oceans occupy 70% of the world's surface, and play an important role in global carbon and nitrogen cycles. A huge three dimensional space exists beneath the surface of the ocean in which vast numbers of marine organisms thrive. Marine Biotechnology may be described as the utilization of these biological resources for the benefit of mankind. These benefits include production of useful chemicals, energy and the remediation of environmental problems.

Biotechnology, appeared as a new industry at the end of the 1970's and developed rapidly as a result of advances in the fields of genetic engineering, cell culture, cell fusion and bioreactor technology. In the 1980's 'second generation' biotechnologies such as plant biotechnology, chromosome engineering, protein engineering and bioelectronics were developed. Recently, Marine Biotechnology has begun to attract attention as a new frontier in biotechnology. The environment of the seas and oceans is different from that on land, and the marine environment holds a large number of biological resources, yet to be discovered.

Marine Biotechnology, in the coming years will help to provide new commercial opportunities in addition to helping to solve environmental problems. Bioremediation of oil spills is one example demonstrating the usefulness of marine organisms in pollution control. In addition, major efforts are under way, aimed at the more serious problems of global warming and climate change.

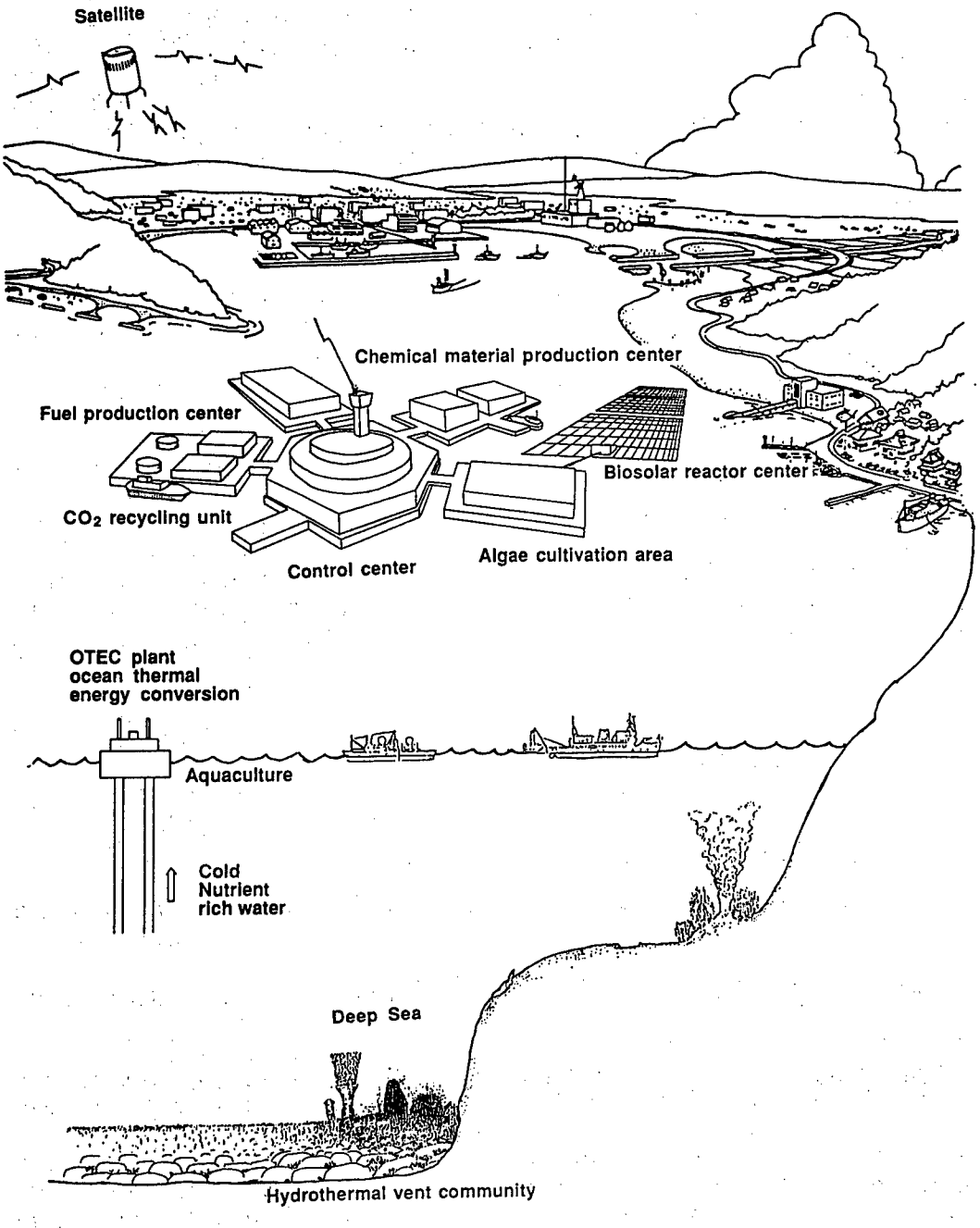

Fig.1 Total ocean utilization system

Available Bioresources

A description of Marine Biotechnology should not be limited to utilization of marine bacteria using molecular genetics but to the utilization of all available bioresources in the oceans. This includes marine viruses, which may be developed into DNA cloning vectors, bacteria as well as microalgae, diatoms, zooplankton, macroalgae, seaweed and invertebrates. In addition, shellfish, crustacea and fish may be looked upon a resources for marine biotechnology. The majority of species present in our oceans have not yet been discovered, and in the case of marine bacteria, many organisms present in seawater can be observed but cannot be cultured in the laboratory. In addition, many species of fish cannot be kept in captivity. Thus we require new methods for the collection, culturing and maintenance of marine organisms.

Research Strategies for Marine Biotechnology

The completion of successful marine biotechnology projects involves careful planning and the initial design of an effective research strategy. The interaction of academia, industry and government towards a common goal should be carried out using a multidisciplinary approach. A research framework upon which to base more detailed plans is outlined in Table 1.

The most important initial step in any marine biotechnological process is to identify the biological resources which be used for a specific purpose. This often involves extensive screening for a desired function. For example isolation of plant growth regulators from marine cyanobacteria [2-4] or the isolation of antibiotics from marine photosynthetic bacteria [22]. These screening procedures are applicable not only to prokaryotes but to screening higher organisms for useful functions. For example, screening of seaweeds suitable for cell culture and establishment of recombinant DNA techniques. Screening of sponges and other marine invertebrates for production of pharmaceuticals. In addition, new types of shell fish, crustacea and fish may be screened for their suitability for aquaculture and open water fish farming.

Following the classical improvement programs which have been successfully employed for the improvement of terrestrial organisms such as wheat and penicillin mould, improvement strategies will ultimately lead to the improvement of organisms found during the initial screening procedures. Classical selection pressure techniques may be used for the selection of microbial strains producing greater quantities of the desired product or which grow faster under laboratory conditions. Chemical mutagenesis and further screening may also be employed. In addition, and of key importance is the development of suitable genetic engineering techniques. For example, in the case of techniques which are available for gene transfer in freshwater photosynthetic bacteria, do not work for marine species of photosynthetic bacteria [23]. Therefore techniques were developed for marine species of *Rhodobacter* with the aim of improving hydrogen photoproduction [24,25]. In general, recombinant DNA techniques for marine organisms are vastly inferior to those available for terrestrial organisms. However, headway is being made in the area of transgenic fish. New methods for direct gene transfer using sonication have recently been developed.

Table 1. Research Strategy for Marine Biotechnology

1) Screening of useful marine organisms;

   bacteria, microalgae, seaweeds, invertebrates and vertebrates.

2) Genetic improvement of target organisms;

   selection pressure, mutagenesis and genetic manipulation techniques followed by further screening.

3) Production of useful chemicals from marine organisms;

   pharmaceuticals, foods, chemicals, cosmetics.

4) Elucidation and application of novel functions of marine microorganisms;

   microbes resistant to heat, cold, pressure, high salt conditions. Application of enzymes and molecular components involved in for example; photosynthesis, nitrogen fixation, metal recovery, buoyancy, luminescence, magnetic sensitivity.

5) Provision of marine biotechnology support systems;

   marine infrastructure for fish farming, algal culture, research, remote sensing from space, down stream technology.

6) Understanding of marine ecosystems and application of this information to global environmental problems;

   ecology of deep sea environments, ecology and application of oil degrading bacteria. The Carbon cycle in marine ecosystems and prevention of global warming. Red tide organisms and factors inducing red tides and other marine algal blooms.

7) Construction of a multi function general ocean utilization system;

   off shore infrastructure with facilities for aquaculture of fish and algae. Mass cultivation of marine microorganisms. CO_2 deposition in the deep sea, CO_2 recycling.

Genetic Improvement of target organisms.

To attempt to increase the usefulness of marine organisms, gene manipulation technology should be developed. We have carried out a program aimed at the genetic improvement of marine cyanobacteria by establishing gene transfer techniques. Such techniques have already been established for fresh water cyanobacteria, *Synechococcus* R2 [8], *Synechocystis* PCC6083 [9] other filamentous cyanobacteria [10] and the marine cyanobacterium, *Synechococcus* PCC7002 [11]. These species are all able to naturally take up exogenous DNA. However, many marine species of cyanobacteria lack this useful property and it was therefore necessary to establish alternative gene transfer techniques for biotechnologically useful strains.

a) Transformation of marine cyanobacteria

Widely applicable gene transfer techniques for marine cyanobacteria have only recently been reported [6]. Previously, gene transfer was only available for one strain, *Synechococcus* PCC7002, which is also naturally transformable. Recently, this laboratory has attempted to establish gene manipulation technology for a marine cyanobacterium, *Synechococcus* sp. NKBG042902 which produces glutamate and novel plant growth regulators [13,2]. Initially, with the aim of constructing shuttle vectors, characterisation of the four endogenous plasmids of this species was carried out. The smallest plasmid, pSY11 (2.3 kb), was used to construct a shuttle vector pUSY02. This was constructed from the *Escherichia coli* cloning vector pUC19 and pSY11, since this plasmid has a high copy number and several convenient restriction sites. pUSY02 could transform marine *Synechococcus* sp. NKBG042902-YG1116, which had been cured of its two smallest endogenous plasmids by treatment with ethidium bromide. This shuttle vector could also transform the fresh water cyanobacterium *Synechococcus* R2 although shuttle vectors developed for *Synechococcus* R2 could not transform the marine *Synechococcus* . For genetic transformation the $CaCl_2$ treatment method which is normally used for *E.coli*, was effective. On the other hand, the freshwater species *Synechococcus* R2 was not transformed by this method.

Recently, the characterization of plasmid, pSY10, a second small endogenous plasmid from *Synechococcus* sp. NKBG042902 was reported [14]. The copy number of pSY10 (2.7 kb) increased from 50 to 250 copies per chromosome when the salinity of the medium increased from 0% NaCl to 3% NaCl . Thus shuttle vectors which contain the replication and copy number control regions of this plasmid may also have their copy numbers controlled by changing the salinity of the medium. This may subsequently be linked to controlled expression of foreign genes in appropriate strains of marine cyanobacteria. Experiments are currently under way to localize the replication origin of pSY10 and to determine the complete nucleotide sequence of this plasmid. A comparison of the pSY10 and pSY11 replicons may also provide information concerning the molecular mechanism of sodium chloride mediated copy number increase.

b) Transformation by electroporation.

Electroporation was used to transform the marine cyanobacterium *Synechococcus* sp. NKBG042902. Conditions were optimized so that the time needed for transformation was reduced [6]. Previously electroporation has only been reported for the filamentous freshwater cyanobacterium *Anabaena* [15]. Electroporation therefore seems to be a useful strategy for gene transfer of both filamentous and unicellular cyanobacteria. In principle, gene transfer using electroporation should be applicable to a wide variety of species although optimization of electroporation parameters will be required for each strain.

c) Transformation by the particle gun method

The use of DNA coated microprojectiles in association with a particle gun is a well established method of transformation [10]. The DNA coated particles are introduced into the cells by an explosive release of high pressure air or gunpowder using a modified 'particle gun'. This method was originally applied to the genetic transformation of plant cells [17]. We have recently used this method for transformation of marine cyanobacteria using the magnetite (Fe_3O_4) particles as DNA carriers [18]. Particles of Tungsten and Gold are normally used as DNA carriers and are often in the 0.1-0.6 μm size range (mean particle size). This limits the amount of DNA which can be immobilized onto these particles due to the surface area to volume ratio. In addition, such large particles are unsuitable for use with small prokaryotic cells such as cyanobacteria. Magnetite particles which are synthesized by magnetic bacteria [21] occur in the 500-1000Å size range and are therefore of a more suitable size range for bombarding prokaryotic cells. In addition, magnetite particles isolated from magnetic bacteria are coated with a biological membrane which also contains proteins. This increases the quantity of DNA which can be immobilized onto the surface of the particles. Bacterial magnetite particles were successfully introduced into cyanobacteria by this method.

d) Conjugative gene transfer.

Conjugative gene transfer has been reported for the fresh water filamentous cyanobacterium, *Anabaena* PCC7120. A conjugation system mediated by *E. coli* is used because no conjugative plasmids have been observed so far in cyanobacteria. A shuttle vector or 'cargo vector' was constructed from a *Nostoc* sp. endogenous plasmid and a mobilizable plasmid which can replicate in *E. coli* . Under triparental conjugation conditions (cargo vector, conjugal plasmid and helper plasmid), the cargo vector can be introduced into *Anabaena* from *E. coli*. This method has also been used with *Freymiella diplosiphon* [19] in order to investigate the mechanism of complementary chromatic adaptation. In addition, conjugative transfer of broad host range vectors, rather than shuttle vectors, has recently been reported for *Synechocystis* PCC6803 [19]. The applicability of gene transfer by conjugation, specifically in marine cyanobacteria, has also been demonstrated [20]. Gene transfer

was successfully achieved for three different genera, *Synechococcus*, *Synechocystis* and *Pseudanabaena*. Conjugation was carried out using a mobilizable transposon (Tn*5*) carrying plasmid, pSUP1021. Transconjugants were observed in all marine cyanobacteria tested. The broad host range vector pKT230 (IncQ) was also successfully transferred to *Synechococcus* sp. NKBG15041C, and could replicate as an autonomous replicon. A maximum transfer efficiency of 5.2 x 10-4 transconjugants / recipient cell was observed, when matings were performed on low salinity (0.015 M NaCl) agar plates.

Production of Useful Chemicals

Recently, application of marine microalgae has received much attention. The most apparent of the many advantages of using marine microalgae, is the wide availability of seawater, since many regions of the world suffer from lack of fresh water. Seawater contains minerals and salts which are essential for the growth of photosynthetic microorganisms. For several years, marine microalgae have been isolated from the coastal areas of Japan, and applied to the production of chemicals and energy, in this laboratory. Among more than 300 strains of marine photosynthetic microorganisms, several strains have been isolated which produce biologically active compounds. The specific application of individual isolates is discussed below.

a) Fatty acid production by marine microalgae.

The fatty acid composition of marine microalgae was studied under various environmental conditions. The most common fatty acids were palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2). One strain, a marine *Chlorella* species NKBG 942401 was found to contain γ-linolenic acid. γ-linolenic acid was present in the cells mainly in the form of galactolipid [1].

b) Plant growth regulators

Extracts of marine cyanobacteria *Synechococcus* sp. NKBG042902 were found to promote plantlet formation by somatic embryogenesis in carrot cells. Somatic embryogenesis stimulating factors in this extract were capable of inducing somatic embryo like structures and plantlets from non embryogenic and embryogenic cells [2]. The effect of the extract on the developmental stages, induction, maturation and germination, in carrot somatic embryogenesis was investigated. A high molecular weight, nondialyzing fraction, separated from the *Synechococcus* extract, possessed enhanced plantlet formation promoting activity [3]. An improved artificial seed system using the nondialysate was developed. The germination frequency of the artificial seeds containing nondialysate at a concentration of 100 mg/l was 94% whereas that of artificial seeds without the nondialysate was 35% [4]. The active substance, a polysaccharide, was shown to promote germination of carrot somatic embryos and has been purified to homogeneity. SDS-PAGE analysis showed this germination promoting factor (GPF) to be a single band. Purified GPF contained,

xylose, glucose and galactose, but did not contain peptides.

c) Amino acid production

The marine cyanobacterium *Synechococcus* sp. NKBG040607 was found to excrete glutamate at a high rate, 82.6 % of the total amino acid production being glutamate. Immobilized cells of *Synechococcus* sp. continuously produced glutamate from nitrate under light irradiation [5].

d) Bioreactor systems

Marine microalgae (green algae and cyanobacteria) are being paid much attention due to their high potential for producing various biologically active compounds. However, an inherent problem in microalgal cultivation is low cell density, which consequently results in low productivity. To solve this problem, novel bioreactor systems suitable for high density culture have been developed for cyanobacteria [6]. With the increase in number of processes utilizing marine microalgae, the requirement for a precise system for monitoring their concentration and physiological state is also increasing. A versatile and precise on-line monitoring system for measuring viable cell numbers of marine cyanobacteria, based on the phycobiliprotein fluorescence was developed [7].

Elucidation of Novel Functions of Marine Organisms.

As an example, the migratory nature of several marine fish will be discussed. Migration behavior is thought to be due to the detection of the geomagnetic field by sensors within the body of the fish. An important component of this sensor is biogenic magnetite. This laboratory has been engaged in the study of magnetite biomineralization. Biogenic magnetite was originally identified as a strengthening mineral in the radula teeth of chitons (*Mollusca, Polyplacophora*). In addition, magnetic particles have also been found in the honey bee and also in higher animals such as the pigeon and in migratory fishes. This magnetite is involved in the sensory perception of the geomagnetic field and in the recognition of direction and location. Although biomineralization of magnetite is clearly important from an ecological and evolutionary standpoint, the molecular mechanisms by which magnetite crystals are formed remain unknown. In order to begin to try and understand such mechanisms we have chosen as a model organism for magnetite biomineralization the freshwater magnetic bacterium *Aquaspirillum* sp. AMB-1. Genetic engineering techniques have been developed for this bacterium and genes involved in the production of biogenic magnetite have been isolated [26]. Magnetic bacteria synthesize intracellular particles of magnetite (Fe_3O_4), which are aligned in chains and enveloped by a membrane. These structures, known as magnetosomes, impart a magnetic dipole to the bacterial cell which is then sensitive to externally applied magnetic fields. The ecological significance of these biological magnets remains unclear, although it has been proposed that sensitivity to the geomagnetic field allows oxygen sensitive magnetic bacteria to swim downward into oxygen poor sediments, their preferred habitat.

Isolation of these genes should help us to clone genes responsible for magnetite production in marine migratory fishes. When such processes have been isolated, possible applications may be considered.

Understanding Marine Ecosystems and Application to global environmental problems.

Recently an understanding of the nature of deep sea ecosystems has been improving and it has become clear that nutrient levels of water from the deep ocean are much higher than sea water near the surface. Thus by pumping sea water up from deeper levels it may be possible to 'fertilize' the surface waters an increase the growth efficiency of microalgae using such water. In addition this may be combined with the growth of cyanobacteria for carbon dioxide removal technologies. For example, the high carbon dioxide emissions from fossil fuel power stations may be reduced if absorbed and utilized by marine microalgae. The economics of such processes will depend on the value of the cyanobacterial products and on whether or not carbon taxes will be imposed for CO_2 emitting industries in the future.

In order to develop technology useful for growing marine photosynthetic organisms for carbon dioxide renewal we have developed photobioreactors for the high density culture of marine cyanobacteria [27]. In addition, we are currently investigating the growth of marine coccolithophorid algae for increased CO_2 recycling by calcite production.

Summary and Future Trends

In the past several years, this laboratory has been involved in establishing multidisciplinary techniques for the biotechnological utilization of marine microalgae. A variety of recombinant DNA methodologies have been developed as well as suitable photobioreactor technology and bioprocess engineering. Recently , with increasing stress on environmental conservation, future applications will involve marine microalgae in carbon dioxide renewal processes which also produce useful chemical products. The research strategies outlined in this presentation are applicable to other organisms as well as microalgae, there successful application will lead the development of new and rewarding technologies of the future.

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B-4-3

GENE MANIPULATION FOR ANIMAL INDUSTRY

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ABSTRACT

Infectious diseases are still one of the major problems of domestic animals. In this paper, the development of a novel live recombinant rinderpest vaccine (RRV) is introduced as one example applying gene manipulation to animal industry. Cattle in Africa, Middle East and South Asia are now under the threat of rinderpest, a highly contagious and mortal disease caused by rinderpest virus (RV). Although attenuated RV vaccine is widely used, it is not fully effective in epidemic areas due to the thermoinstability of the vaccine and the lack of a cold chain in these areas. Thus, the development of thermostable rinderpest vaccine is urgently needed. The newly developed RRV is a recombinant vaccinia virus expressing rinderpest virus hemagglutinin gene. It is as heat-stable as the parental vaccinia virus, which was used for the global eradication campaign of smallpox. Furthermore the RRV keeps low neurovirulence of the parental virus. The efficacy has been demonstrated in cattle, the target animal of the vaccine. The RRV has fulfilled essential conditions proposed by the expert consultation on requirements for vaccinia-rinderpest recombinant vaccines held by Office International des Epizooties (OIE) in Paris in 1989. The OIE meeting also proposed the guideline for field trials. Lastly, we will discuss the problems and perspectives of the development of live recombinant vaccine in the future.

INTRODUCTION

In the human, acute infectious diseases are now not so horrible as before due to the availability of vaccines and antibiotics although persistently infecting viruses such as human immunodeficiency viruses are menace to us. In economic animals, however, infectious diseases are still the largest

threat inflicting damage on them as they have relatively short life cycles and their living environment is worse than that of the human. Thus, the development of vaccine holds an important position in animal industry. Gene manipulation has now become an essential and rather general technology for vaccine development. In this paper, we will review our work on the rinderpest vaccine as an example of vaccine development by employing gene manipulation, and discuss the perspective of live recombinant vaccines in the future.

BACKGROUND OF RINDERPEST

Rinderpest is an acute febrile and contagious viral disease with high morbidity and mortality affecting mainly cattle and buffaloes. Because of recent outbreaks of rinderpest throughout Africa, the Middle and Near Easts, and South Asia, eradication campaigns including the pan-African Rinderpest Campaign (PARC) have been initiated in these countries[1]. There is no dispute about the seriousness of the disease. It causes high fever and inflammation and lesions of the respiratory and alimentary tracts. It kills affected animals in few days and shows fatality higher than 50%. The current rinderpest vaccine which consists of attenuated live rinderpest virus (RV) is highly effective in protection but is not heat-stable for wide use in the above-mentioned countries where the cold chain system is not always available. Therefore, we undertook the development of a novel heat-stable rinderpest vaccine.

A NOVEL VACCINE USING RECOMBINANT VACCINIA VIRUS

In 1980 the World Health Organization (WHO) declared the eradication of smallpox all over the world. Accordingly, the smallpox vaccine is no longer needed by the general population. Especially, the high heat stability of freeze-dried smallpox vaccine as well as its ease of administration contributed to the achievement of the eradication campaign. Now the technology has been established to use smallpox vaccine, which consists of vaccinia virus, as a vehicle to harbor and express heterologous genes which code for the immunologically pertinent antigens of other infectious disease agents[2, 3]. Inoculation of the resultant recombinant vaccinia virus is able to induce immune responses targeted toward both vaccinia and the heterologous antigens. The recombinant virus is considered to keep most of the characteristics of the parental vaccinia virus. We have undertaken to construct a heat-stable rinderpest recombinant vaccine (RRV) using vaccinia virus as a vector in expectation that the resultant RRV may also be as heat stable as the parental vaccinia virus. First, the cDNA coding for the RV hemagglutinin protein was cloned and sequenced[4], and thereafter was

inserted into an attenuated vaccinia virus, LC16mO[5]. The RRV induced strong anti-hemagglutinin antibody responses and protective immunity in rabbits[6]. In order to further augment the efficacy of the RRV, we modified the promoter regions of the RRV. This RRV was found to express more RV hemagglutinin and induced ten-times stronger protective immunity in terms of 50% protective dose than the original RRV[7]. Thus the modified RRV was used in the following cattle experiments. When the lyophilized samples of the RRV was heat-treated either at 37° C or at 45° C, a slight decrease in virus titer not exceeding 1/10 of original levels was observed after 1 month of heat treatment, implicating high heat stability of the RRV. Neurovirulence of the RRV as determined in mice, rabbits and squirrel monkeys was as low as that of the parental vaccinia virus. Based on these results, the efficacy of the RRV was examined in cattle, the target animal. Vaccination experiments were conducted in cooperation with Indian Veterinary Research Institute using Hill bulls of Indian breed[8] and with AFRC Institute for Animal Health, Pirbright Laboratory, U. K. using Friesien cross Aberdeen calves[9]. Essentially the same results were obtained in the two Institutes. The inoculation of 10^8 pfu of RRV completely protected the animals against a lethal dose of RV; fever, diarrhea, stomatitis and nasal and ocular discharge were absent in RRV-vaccinated animals whereas all control animals manifested these symptoms and died within 8 days. 50% protective doses determined by mortality rate were 10^4 pfu in the Hill bulls and 10^5 pfu in Friesien cross Aberdeen calves. For the purpose similar to ours, two other groups reported the results relating to the development of RRV. Barret *et al.* of the British group[10] reported the construction of RRV expressing RV fusion protein gene in WR strain of vaccinia virus, and showed its efficacy in cattle and pigs. The U.S. group of Yilma *et al.*[11] reported that the inoculation of a vaccinia virus double recombinant expressing the fusion protein and hemagglutinin protein genes of RV protected cattle against rinderpest.

OIE MEETING

The Expert Consultation on Requirements for Vaccinia-Rinderpest Recombinant (VRR) vaccines met at the Office International des Epizooties (OIE) Headquarters from 21 to 24 August 1989[12]. One of the authors (K.Y.) attended the meeting as a participant. The report states its purpose as follows: "Recent advances in the development of vaccinia-rinderpest recombinant viruses have amply demonstrated the possibility of an entirely new generation of rinderpest vaccines provided that safety and efficacy criteria can be safely met. " The followings are part of the items discussed

in the meeting: risks associated with the use of RRV, efficacy requirements, and guidelines for the field release of RRV vaccines. At any way, it is very meaningful that an international meeting was held with regard to the safety and efficacy of a recombinant virus vaccine. Consultation with the recommendations and guidelines of OIE meeting would help to avoid potential problems accompanied by vaccine development.

PROBLEMS AND PERSPECTIVE OF THE ANIMAL VACCINE DEVELOPMENT USING LIVE RECOMBINANT VIRUSES

Gene technology is used at various stages of vaccine development and now is one of essential techniques in this area. The outline of viral vaccine development is summarized in Figure 1.1) Cloning and sequencing the virus genome and elucidation of the function of its genes. 2) Modification of virus genome to generate a virus with favorable characteristics as a live vaccine. 3) Construction of live recombinant viruses, bacteria and other organisms expressing foreign antigen. 4) Cloning of the viral gene coding for protective antigen and its expression using various host/vector systems. 5) Mass production of the antigen for component vaccines.

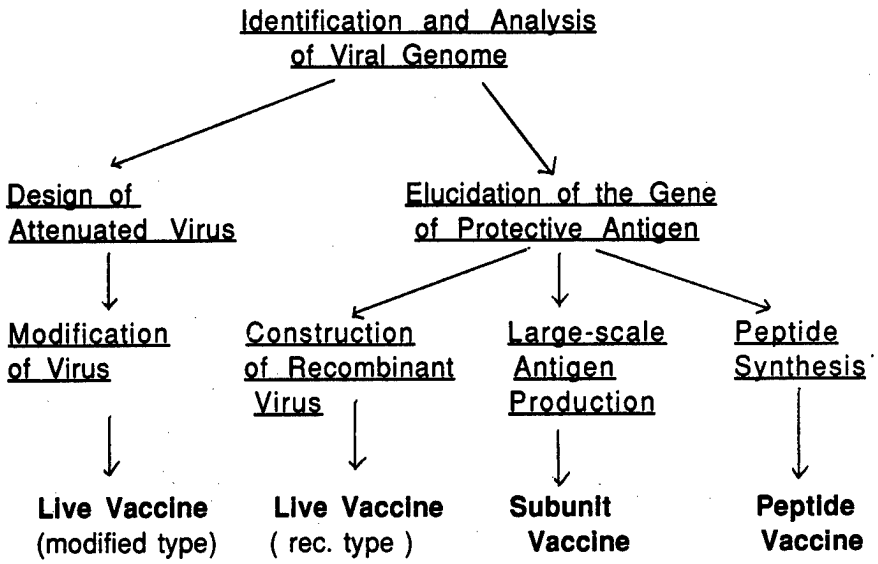

Fig. 1 Outline of viral vaccine development using gene manipulation

The application of gene technology to vaccine development has the advantage that a large scale growth of pathogenic viruses is not necessary to obtain the antigens. There are three key factors for vaccines; 1) efficacy, 2) safety and 3) economy. In order to fulfill these conditions, elaboration of the molecular design of vaccines are essential regardless of whether it is a live vaccine or component vaccine. Live vaccines are usually more effective and cheaper than component vaccines. On the other hand, component vaccines are safer than live vaccines. Live vaccine made by gene engineering are roughly divided into two categories: 1) the achievement of attenuation by modifying part of gene(s) which is responsible for the virulence of viruses (live modified vaccine), and 2) the construction of recombinant viruses expressing the protective antigen of target virus by the use of an attenuated virus as a vector (live recombinant vaccine). For the construction of a live modified vaccine, it is essential to elucidate the viral gene(s) responsible for virulence. Thymidine kinase is known as one of such genes, and deletion of the function of the gene is known to lead to the reduction of virulence either in pseudorabies virus of pig and vaccinia virus. In the case of vaccinia virus, however, the loss of the function of thymidine kinase gene results in a marked reduction of viral replication leading to the loss of vaccine effect. Therefore, when a recombinant virus is constructed it is rather preferable to insert a foreign gene into the gene lesion which is not involved in viral replication. From this point of view, the use of attenuated vaccinia virus as a vector is essential. In our study on the RRV, we employed this strategy, that is, RV hemagglutinin protein gene was inserted into the hemagglutinin protein gene of LC16mO, an attenuated vaccinia virus. In the future, it is possible to construct an ideal vaccinia virus vector with highly attenuated nature and ample activity of replication, if all genes responsible for these functions are known. In this regard, it is interesting to note that a vaccinia virus gene related to complement system proteins (*ps/hr* gene) was found to be responsible for the regulation of plaque size and host range of the virus but was not involved in the neurovirulence[13]. The augmentation of the expression of foreign antigen also contributes to the improvement in efficacy, safety and economy. As shown in the case of RRV, the modification of promoter region augmented the amount of antigen expression by ten times, resulting in the decrease of vaccine dose to 1/10 of the unmodified vaccine for successful immunization in rabbits. Recombinant vaccinia viruses usually possess a high activity to induce cell-mediated immunity. For example, it was suggested that the inoculation in sheep of a recombinant vaccinia virus expressing bovine leukemia virus (BLV) envelope glycoprotein suppressed BLV replication via the augmentation of cell-mediated immune

responses[14], suggesting the possibility to utilize the virus as a therapeutic vaccine for retrovirus infection. One of the problems of recombinant vaccinia virus is that it sometimes fails to induce sufficient humoral immunity although it usually induce strong cell-mediated immunity. This defect may be also overcome by the construction of a recombinant virus which possesses a proper replicating activity and an augmented expression of target antigen. Several virus and bacteria vectors other than vaccinia virus have been also used as an expression vector of foreign antigens, including fowlpox virus and bacille Calmette-Guerin (BCG). It is also noted that a field trial aiming at large-scale eradication of rabies using recombinant vaccinia-rabies vaccine has been successfully done in Europe[15]. The results of both rabies and rinderpest suggest a promising future of the practical application of recombinant vaccines. Recombinant vaccinia viruses have the following advantages: 1) being able to induce strong cell-mediated immunity, 2) usable as a multi-valent vaccine, 3) easy to produce and inoculate 4) cheap, 5) heat stable. Safety is the most serious potential problem of the vaccine, but as discussed at the OIE meeting the utilization of an attenuated vaccinia virus as a vector will overcome the problem. Live recombinant vaccines have a special merit when used in a large scale, such as the eradication campaign of rabies and rinderpest.

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C-1-5

MICROBIAL SYNTHESIS AND PROPERTIES OF BIODEGRADABLE PLASTICS

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ABSTRACT

Three types of copolymers, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), poly(3-hydroxybutyrate-co-4-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxypropionate), are produced by *Alcaligenes eutrophus* from various carbon substrates. The microbial polyesters are thermoplastics with biodegradable properties, and the physical properties can be regulated by varying the compositions of copolymers. These bioplastics have attracted industrial attention as environmentally degradable thermoplastics.

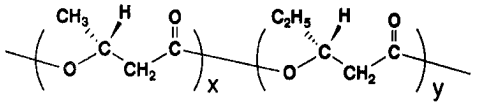
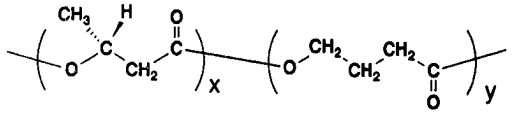
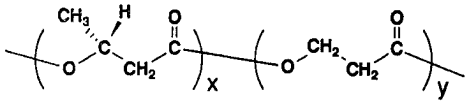
INTRODUCTION

A wide variety of microorganisms accumulate an optically active polymer of (R)-3-hydroxybutyric acid, P(3HB), as an intracellular storage material of carbon and energy (1, 2). Many prokaryotic organisms such as bacteria and cyanobacteria have been found to accumulate P(3HB) up to 80% of their cellular dry weight, when growth is limited by the depletion of an essential nutrient such as nitrogen, oxygen, phosphorus or magnesium. Recently, some bacteria have been found to accumulate copolymers containing (R)-3-hydroxyalkanoate units other than (R)-3-hydroxybutyrate. More recently, copolymers containing 4-hydroxybutyrate unit have been produced by several bacterial strains. The general class of microbial polyesters is called poly(hydroxyalkanoates) (PHA) (1, 2).

These microbial polyesters have attracted much attention as environmentally degradable thermoplastics for a wide range of agricultural, marine and medical applications (3). The microbial polyesters are degradable in soil, sludge or sea water. Some microorganisms such as bacteria and fungi secrete extracellular P(3HB) depolymerase to degrade environmental microbial polyesters and utilize the decomposed compounds as nutrient.

This paper surveys the PHA copolymers produced by *Alcaligenes eutrophus* from various carbon sources and discusses the pathway and its regulation of PHA synthesis. In addition, the biodegradability of PHA products is studied.

Table 1 PHA random copolymers produced by *A.eutrophus* from various carbon sources

Carbon source	Random copolymer
Propionic acid Pentanoic acid	 (R) - 3HB (R) - 3HV
4-Hydroxybutyric acid γ-Butyrolactone 1,4-Butanediol 1,6-Hexanediol 1,8-Octanediol 1,10-Decanediol 1,12-Dodecanediol	 (R) - 3HB 4HB
3-Hydroxypropionic acid 1,5-Pentanediol 1,7-Heptanediol 1,9-Nonanediol	 (R) - 3HB 3HP

PRODUCTION OF P(3HB-co-3HV) COPOLYMER

Table 1 lists the PHA copolymers produced by *A.eutrophus* from various carbon sources. Three different types of random copolymers were produced from various carbon substrates. The copolymers of (R)-3-hydroxybutyrate and (R)-3-hydroxyvarelate P(3HB-co-3HV) have been commercially produced by ICI, UK in a large scale, two-stage, fed-batch fermentation of *A.eutrophus*, feeding propionic acid and glucose as the carbon sources (4). In the first stage, *A.eutrophus* cells grow and multiply in a glucose-salts medium under conditions of carbon and nutrient excess. In the second stage, the phosphate supply becomes depleted and propionic acid is fed. The P(3HB-co-3HV) copolymers are accumulated up to 75% of total dry cell weight in the second stage of phosphate limitation. The total fermentation time is in the order of 110 - 120 hours. The copolyester compositions vary from 0 to 47 mol% 3HV, depending on the ratio of propionic acid and glucose supplied. The biosynthetic pathway of P(3HB-co-3HV) in *A.eutrophus* has been investigated by using ^{13}C-labeled acetic and propionic acids as the carbon sources (5) (Figure 1). The P(3HB-co-3HV) copolymers with a wide range of compositions from 0 to 90 mol% 3HV are accumulated in *A.eutrophus* by using butyric and pentanoic acids as the carbon sources (6). The butyric and pentanoic acids are respectively incorporated into 3HB and 3HV units via acetoacetyl-CoA in the β-oxidation cycle, as shown in Figure 1. The regulation of P(3HB-co-3HV) biosynthesis from butyric and pentanoic acids is of interest because the key regulatory enzyme of polymer synthesis from acetyl-CoA, 3-ketothiolase, is not involved.

The P(3HB-co-3HV) copolymers were produced at 30°C from various

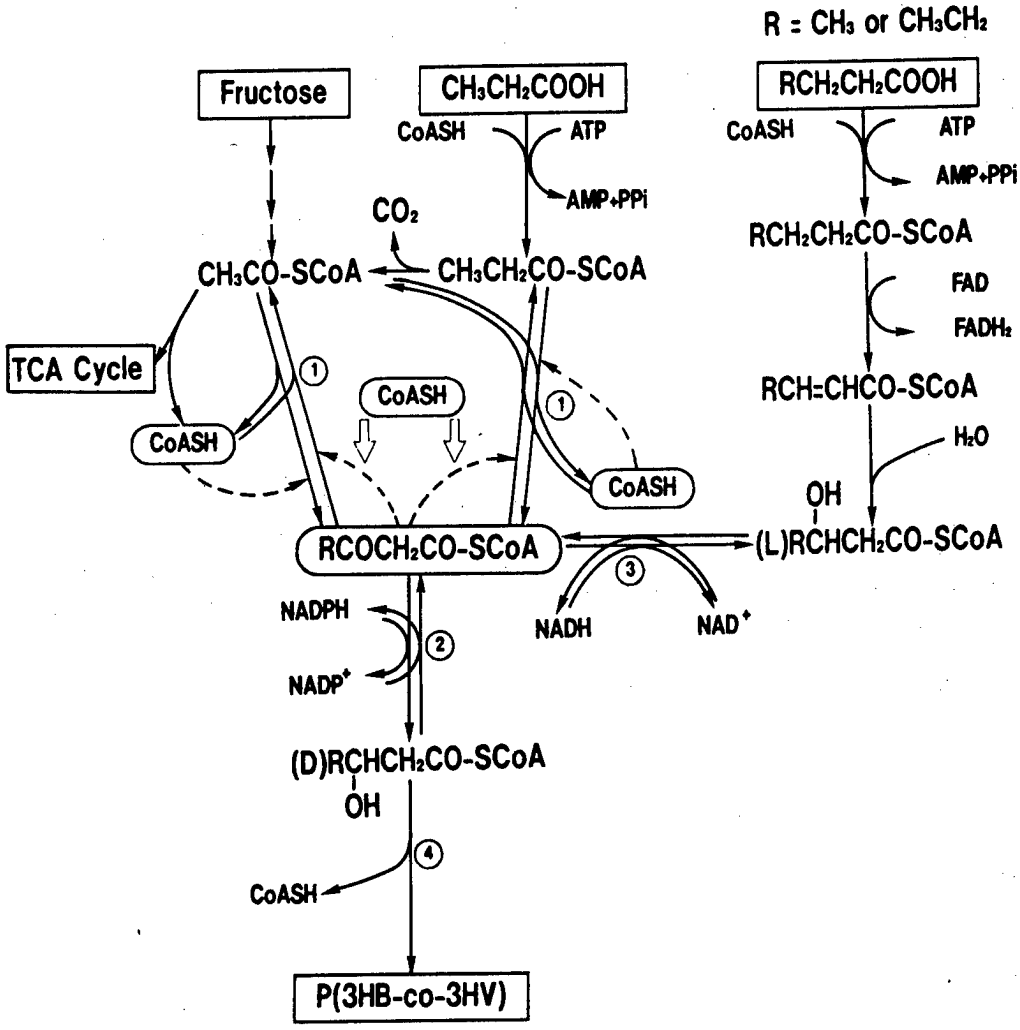


Figure 1 Pathways of P(3HB-co-3HV) synthesis in *A.eutrophus*. (1), 3-Ketothiolase; (2), NADPH-linked acetoacetyl-CoA reductase; (3), NADH-linked acetoacetyl-CoA reductase; (4), P(3HB) synthase. The dotted lines indicate inhibition.

carbon sources by *A.eutrophus* under fed-batch growth conditions. The production of P(3HB-co-3HV) from butyric and pentanoic acids was effective under nitrogen-limited conditions, and the conversion of carbon sources into copolymers was as high as 56 wt% at the C/N mole ratio of 40. In contrast, under nitrogen-excess conditions (C/N<10), the cell growth was good, while the production of P(3HB-co-3HV) from fructose and propionic acid was almost completely inhibited under nitrogen-excess conditions.

PRODUCTION OF P(3HB-co-4HB) COPOLYMER

The copolymers of (R)-3-hydroxybutyrate and 4-hydroxybutyrate P(3HB- co-4HB) are produced by *A.eutrophus* from 4-hydroxybutyric acid, 1,4-butanediol or γ-butyrolactone (7, 8). When 4-hydroxy-butyric acid was used as the sole carbon source, a P(3HB-co-33% 4HB) was produced. The addition of butyric acid in the 4-hydroxybutyric acid culture solution resulted in a decrease in the 4HB fraction. Thus, the copolymer compositions were varied from 0 to 33 mol% 4HB, depending on the carbon substrates supplied in the feed. Recently, we have found that the P(3HB-co-4HB) copolymers with a wide range of compositions from 0 to 100 mol% 4HB are produced by *A.eutrophus* from 4-hydroxybutyric acid in the presence of some additives. When 4-hydroxybutyric acid, citrate and ammonium sulfate were fed as the mixed substrates, P(3HB-co-4HB) copolymers with compositions of 70 - 100 mol% 4HB were produced.

Figure 2 shows a schematic pathway of P(3HB-co-4HB) biosynthesis in *A.eutrophus*. 4-Hydroxybutyryl-CoA is first formed from 4-hydroxybutyric acid in the *A. eutrophus* cells. A portion of 4-hydroxybutyryl-CoA is then metabolized into (R)-3-hydroxybutyryl-CoA via acetoacetyl-CoA in the β-oxidation cycle. A random copolymer of 3HB and 4HB units is synthesized by the copolymerization of (R)-3-hydroxybutyryl-CoA with 4-hydroxy-butyryl-CoA under the actions of P(3HB) polymerase. When $(NH_4)_2SO_4$ and citrate are added to *A.eutrophus*, acetoacetyl-CoA from 4-hydroxybutyryl-CoA is metabolized into acetyl-CoA rather than into (R)-3-hydroxybutyryl-CoA under growth conditions, resulting in an increase in the 4HB fraction.

The copolymers of (R)-3-hydroxybutyrate and 3-hydroxypropionate P(3HB-co-3HP) are produced by *A.eutrophus*, when 3-hydroxypropionic acid is used as the carbon source (9). The 3HP content is still limited to the range 0 - 7 mol%. The P(3HB-co-3HP) copolymers were also produced from the alkanediols of odd carbon numbers such as 1,5-pentanediol, 1,7-heptanediol and 1,9-nonanediol. In contrast, P(3HB-co-4HB) copolymers were produced from the alkanediols of even carbon numbers such as 1,4-butanediol, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol.

These microbial polyesters are thermoplastics with biodegradable properties (10, 11), and the mechanical properties can be regulated by varying the compositions of copolymers (Table 2).

BIODEGRADATION OF MICROBIAL POLYESTERS

A remarkable characteristic of microbial polyesters is that they are thermoplastic with environmentally degradable properties. The

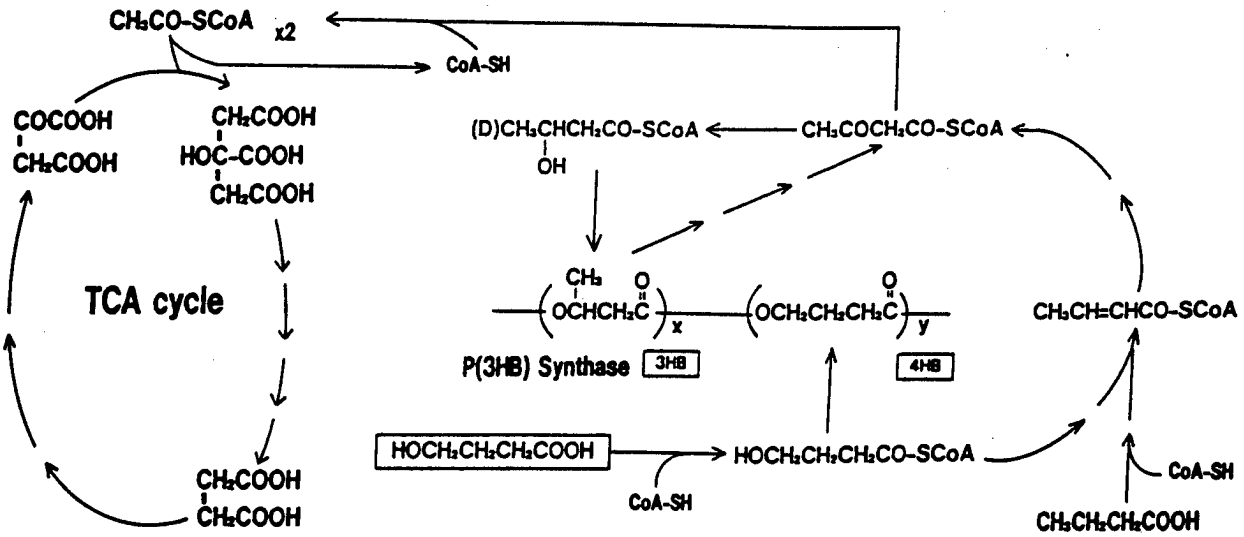


Figure 2 Pathways of P(3HB-co-4HB) and P(3HB-co-3HP) synthesis in *A.eutrophus*.

Table 2 Physical and mechanical properties of P(3HB-co-4HB) films at 23 °C

Composition, (mol%)		Crystal-linity (%)	Density (g/cm^3)	Stress at yield (MPa)	Elonga-tion at yield (%)	Tensile strength (MPa)	Elonga-tion to break (%)
3HB	4HB						
100	0	60 ± 5	1.250	-	-	43	5
97	3	55 ± 5	n.d.	34	4	28	45
90	10	45 ± 5	1.232	28	5	24	242
84	16	45 ± 5	1.234	19	7	26	444
56	44	15 ± 5	n.d.	-	-	10	511

Table 3 Decrease in the thickness of P(3HB) films in various environments at 25 °C

Environment	Rate of degradation (μm/week)
Aerobic sewage	7
Soil	5
Sea water	5

biodegradability of PHA products has been studied in environments such as soil, aerobic sewage and sea water. The processes of biodegradation were analyzed by monitoring the time-dependent changes in weight loss (erosion), molecular weights and mechanical strength of films, plates and fibers of microbial polyesters. The all samples exposed in environments were degraded via surface erosion. The rates of surface erosion of P(3HB) film in various environments at 25°C are given in Table 3. The rate of surface erosion in sea water was almost independent of the copolymer compositions of P(3HB-co-3HV) and P(3HB-co-4HB) samples. In contrast, the erosion rates in soil and aerobic sewage were strongly dependent of copolymer compositions and decreased in the order P(3HB-co-4HB) > P(3HB) > P(3HB-co-3HV). These results suggests that extracellular P(3HB) depolymerases from various bacteria have different specificities on the degradation of microbial polyesters.

An extracellular P(3HB) depolymerase was purified from *Alcaligenes faecalis* which had been isolated in aerobic sewage (12). In a previous paper (13), we showed that the rate of enzymatic degradation of PHA films was faster by two or three orders of magnitude than the rate of simple hydrolytic degradation. The enzymatic degradation occurred at the surface of PHA film and the rate of surface erosion decreased in the order P(3HB-co-4HB) > P(3HB) > P(3HB-co-3HV).

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C-2-2

CHARACTERISTICS AND APPLICATION OF THERMOSTABLE ENZYMES

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ABSTRACT

We have cloned the genes of thermostable D-amino acid aminotransferase, alanine racemase, leucine dehydrogenase, alanine dehydrogenase and phenylalanine dehydrogenase from thermophilic bacterial strains in *E. coli*, constructed the enzyme overproducers, and characterized the enzymes. The thermostable alanine racemase is resistant to not only heat but also proteolysis. It retained considerable activity even after proteolytic cleavage into two peptides. We generated a mutant which was genetically engineered to produce two polypeptide fragments corresponding to the domains. The fragments associate with each other to form an active structure, which was termed "fragmentary form" and shows about 40% of the specific activity of the wild-type enzyme. The CD study of the fragmentary enzyme showed that the secondary structure of the form is closely similar to that of the wild-type enzyme. The two mutant enzymes of D-amino acid aminotransferase in which the active lysine residues were replaced by arginine and alanine residues were produced and characterized. We have developed a procedure for the synthesis of various D-amino acids by means of thermostable D-amino acid aminotransferase, alanine racemase, and L-alanine dehydrogenase. D-Amino acids are produced from the corresponding keto analogues with D-alanine in the system, and optically pure D-enantiomers of glutamate, methionine, leucine, histidine, valine, tryptophan, arginine and several others were synthesized with molar yields of more than 90%. We have established also an *in situ* method for determination of stereospecificity of hydrogen transfer catalyzed by NAD-dependent dehydrogenases by means of amino acid racemase with broad substrate specificty and alanine racemase. We have construction a chimeric thermostable NAD-dependent amino acid dehydrogenase, which shows very broad substrate specificity, derived from thermostable leucine dehydrogenase and phenylalanine dehydrogenase.

INTRODUCTION

Enzymes are characterized by their high catalytic efficiency, and high structural, stereo- and reaction specificities. They are applicable to the production of useful optically

active compounds. However, they are generally susceptible to heating and acid, alkali and organic solvent treatments. We have demonstrated various bacterial thermostable pyridoxal enzymes, and shown their potentialities in the application to enantiomer and diastereomer synthesis. We here describe characterization of thermostable enzymes, D-amino acid synthesis by means of thermostable D-amino acid aminotransferase, alanine racemase and L-alanine dehydrogenase, an *in situ* method for determination of stereospecificity of hydrogen transfer catalyzed by NAD-dependent dehydrogenases by means of amino acid racemase with broad substrate specificty and alanine racemase, and construction of a chimeric thermostable NAD-dependent amino acid dehydrogenase, which shows very broad substrate specificity, derived from thermostable leucine dehydrogenase and phenylalanine dehydrogenase.

I. D-Amino Acid Aminotransferase

D-Amino acid aminotransferase (EC 2.6.1.21) catalyzes transamination between various D-amino acids and α-keto acids, and occurs in bacteria, particularly in the genus *Bacillus*, and higher plants. We have isolated a thermophile which grows in a medium containing D-amino acids as a nitrogen source and identified as a new *Bacillus* species. The organism (*Bacillus* sp. YM-1) showed a very high activity of D-amino acid aminotransferase. The enzyme was purified to homogeneity from cell-extracts of YM-1. It has a molecular weight of about 62,000, and is composed of two subunits identical in molecular weight (30,000). The gene of the enzyme from YM-1 was cloned into *E. coli* C600 cells with the vector plasmid pBR322. The clone cells carrying the plasmid of 4.3-kb DNA (pICT113) produced the enzyme abundantly: its amount corresponds to about 10% of the total cellular proteins. The enzyme overproduced by the clone cells was purified from cell-extracts about 10-fold to homogeneity in a 60% yield by heat treatment and another three steps. The complete primary structure of the enzyme and the position of the active site lysyl residues were determined from the nucleotide sequence of the gene and the amino acid sequence of tryptic peptides. We have studied the stereospecificity of the transamination reaction catalyzed by the enzyme. The C-4' of the cofactor was protonated and deprotonated on the *re* face in the reaction catalyzed by the D-amino acid aminotransferase of *Bacillus* sp. YM-1 with an amino donor, D-alanine. This is the first evidence for the opposite stereospecificity to those of all other aminotransferases, and PLP dependent enzymes catalyzing an abortive transamination so far studied. This fact and the high catalytic efficiency of D-amino acid aminotransferase lead to a conclusion that there is no catalytic advantage to be gained from one or the other side of the cofactor-substrate imine for transamination reaction. In contrast, the C-4' protonation of the cofactor occurred on the *si* face in the transamination reaction of D-amino acid aminotransferase when L-alanine was used as a substrate. This suggests that some other basic residue than 145-Lys is probably located at the *si* face to be able to play as a catalytic base for L-alanine. The stereospecificity of the reaction catalyzed by L-branched-chain amino acid aminotransferase of *E. coli* was shown to be identical with that of D-amino acid aminotransferase. This stereospecific identity supports the previous proposal that the amino acid sequence homology between them is significant in the region corresponding to the catalytic sites, and the two aminotransferases can be classified as a group different from the other three

aminotransferase groups.

Lys-145 of the thermostable D-amino acid aminotransferase, which binds PLP, was replaced by Ala or Arg by site-directed mutagenesis. Both mutant enzymes were purified to homogeneity. Their absorption spectra indicated that both mutant enzymes contained PLP bound non-covalently. Even though we did not any activity with either mutant, addition of D-alanine to the K145R mutant enzyme by the standard assay method led to a slow decrease in absorption at 392 nm with a concomitant increase in absorption at 333 nm. This result suggests that the enzyme was converted into the pyridoxamine phosphate form. The amount of pyruvate formed was almost equivalent to that of the reactive PLP in the mutant enzyme. Thus, the K145R mutant enzyme is able to catalyze the half-reaction of transamination slowly .

Exogenous amines, such as methylamine, had no effect on the half-reaction with the K145R mutant enzyme. In contrast, the K145A mutant enzyme neither showed the spectral change by addition of D-alanine nor catalyzed pyruvate formation, in the absence of added amine. However, the K145A mutant enzyme catalyzed the half-reaction significantly in the presence of added amine. These findings suggest that a basic amino acid residue such as lysine or arginine is required at the position 145 to catalyze the half-reaction. The exogenous amines are bound non-covalently to the active site to mimic the active lysine, and play a dual role as a base in the formation of schiff base with pyridoxal phosphate (pyridoxal-P), and in the α-hydrogen abstraction.

II. Alanine Racemase

Alanine racemase (EC 5.1.1.1) catalyzes the interconversion between L- and D-alanine, providing the D- enantiomer for the synthesis of the peptidoglycan of bacterial cell wall. The enzyme occurs ubiquitously in bacteria. We have cloned the gene of thermostable alanine racemase of *B. stearothemophilus* into *E. coli* with a vector plasmid, pICR301 to produce the enzyme abundantly, and purified it. The enzyme has a molecular weight of about 78,000 and consists of two identical subunits (mol. wt., 39,000), which contain one mole of pyridoxal-P per subunit. The entire amino acid sequence of the thermostable alanine racemase has been deduced from the DNA sequence of the gene.

The guanidine hydrochloride (GdnHCl)-induced subunit dissociation and unfolding of thermostable alanine racemase from *B. stearothermophilus* were studied by circular dichroism (CD), fluorescence and absorption spectroscopies, and gel filtration. The overall process was found to be reversible: more than 75% of the original activity was recovered upon reduction of the denaturant concentration. In the range of 0.6-1.5 M GdnHCl, the dimeric enzyme was dissociated into a monomeric form, which was catalytically inactive. The monomeric enzyme appeared to bind the cofactor pyridoxal-P by a non-covalent linkage, although the native dimeric enzyme binds the cofactor through an aldimine Schiff base linkage. The monomer was mostly unfolded with a transition occurring in the range of 1.8 to 2.2 M GdnHCl.

We examined limited proteolysis of the thermostable alanine racemase with subtilisin.

SDS-PAGE showed that the enzyme is composed of two identical subunits with Mr43,000, and it was cleaved into two major fragments with Mr14,000 (fragment, F-1) and 29,000 (F-2). The amino acid compositions of F-1 and F-2 were determined and the result suggests that the enzyme is first cleaved by proteolysis around ^{264}Tyr. The limited proteolysis was monitored at intervals by densitometric determination of the stained protein band in polyacrylamide gel. Approximately 85% of the native protein disappeared after incubation for 72 hr, but about 50% of the original activity remained. This suggests that about 15% of the native enzyme was unchanged, and the activity corresponding to 35% of the original activity was probably derived from the peptide fragments produced. Therefore, it is conceivable that the peptide fragments of the thermostable alanine racemase interact with each other more strongly to form an active structure than those of usual thermolabile enzymes.

Limited proteolysis studies on alanine racemases suggested that the enzyme subunit is composed of two domains. We constructed a mutant gene which tandemly encodes the two polypeptides of the *B. stearothermophilus* enzyme subunit cleaved at the position corresponding to the predicted hinge region. The mutant gene product purified was shown to be composed of two sets of the two polypeptide fragments, and was immunologically identical to the wild-type enzyme. The mutant enzyme, *i.e.* the fragmentary alanine racemase was active in both directions of the racemization of alanine: the maximum velocity (Vmax) was about a half of that of the wild-type enzyme; the Km value was about twice as high. Absorption and circular dichroism spectra of the fragmentary enzyme were similar to those of the wild-type enzyme. A single polypeptide corresponding to each domain was attempted to be expressed separately in *E. coli*, but no protein that is reactive with the antibody against the wild-type alanine racemase was produced. Therefore, it is suggested that the two polypeptide fragments can fold into an active structure only when they are produced, and that they correspond to structural folding units in the parental polypeptide chain.

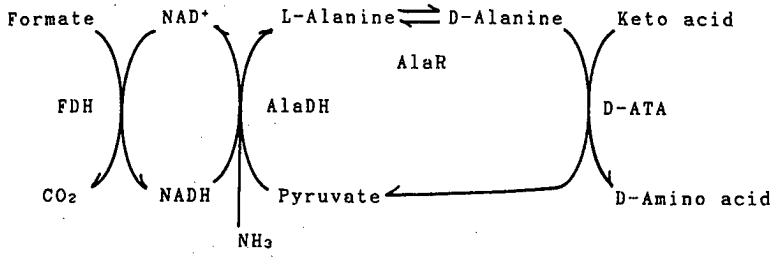
We isolated the large and the small fragments from the fragmentary alanine racemase after denaturation with 6 M GdnHCl. The two fragments were reconstituted and reactivated after the concentration of the denaturant reduced as judged from the result s of gel-filtration analysis and the activity measurement. The spectra obtained by summation of the CD spectra of the isolated subunits were close to those of the native fragmentary enzyme. The lysine residue to which PLP is bound in the wild-type enzyme occurs in the large peptide of the fragmentary enzyme. The absorption spectrum of the large peptide indicates that PLP is not bound to it. The large peptide alone showed no appreciable activity, but it was activated by incubation with the small peptide. These indicate that the two fragments are a unit of the folding, and that both fragments are needed to be folded together in order to form an active structure.

III. Enzymatic Synthesis of D-Amino Acids

We have developed a procedure for the synthesis of D-amino acids on the basis of the stereoselectivity of D-amino acid aminotransferase. Various D-amino acids are produced from D-alanine and the keto analogues, and pyruvate formed is aminated to

L-alanine by alanine dehydrogenase (EC 1.4.1.1), which is also a thermostable enzyme of *E. coli* clone cells carrying the plasmid that contains the dehydrogenase gene from *B. stearothermophilus*. L-Alanine is racemized by alanine racemase to form D-alanine. NADH is regenerated with formate by catalysis of formate dehydrogenase (EC 1.2.1.2) of a *Candida* yeast.

This procedure is based both on the low stereospecificity and very high substrate



Abbreviations are: FDH, formate dehydrogenase; AlaDH, alanine dehydrogenase; AlaR, alanine racemase; D-ATA, D-amino acid aminotransferase

specificity of alanine racemase, the high stereoselectivity and low structural specificity specificity of alanine dehydrogenase. Thus, various D-amino acids are produced from their corresponding keto analogues by the aminotransferase reaction with a consumption of an equimolar amount of ammonia and formate. The reactions of D-amino acid aminotransferase, alanine racemase and L-alanine dehydrogenase are reversible, but the formate dehydrogenase reaction is irreversible. The D-amino acids produced are not racemized by alanine racemase.

The standard reaction mixture contained α-ketoglutarate, sodium formate, NH_4Cl / NH_4OH buffer (pH 8.1), DL-alanine, NAD^+, pyridoxal-P, and the four enzymes (FDH: AlaR: D-ATA: AlaDH=1:1:1:5 in units) in a total volume of 0.5 ml. After incubation at 50°C for various times, an aliquot was mixed with 12% trichloroacetic acid. Glutamate was shown in the supernatant solution with an automatic amino acid analyzer. On incubation of the reaction mixture for 4 h. nearly 100% of the α-ketoglutarate initially added was converted to D-glutamate, whose e.e. was 97%. To optimize the reaction conditions for the production of D-glutamate, we studied the effect of concentrations of substrates and found that the initial rate of D-glutamate production was highest when 0.2 M α-ketoglutarate, 1 M sodium formate, and 1 M NH_4OH were used. The most appropriate ratio of amounts of the four enzymes was FDH: AlaR: D-ATA:AlaDH=1:3:3:5 in units of each enzyme activity.

Under the optimum conditions thus established, the optically pure D-glutamate was produced from α-ketoglutarate in a large scale reaction mixture (30 ml) with a molar yield of about 100%. D-Methionine, D-leucine, and several other D-amino acids were produced with a high yield in the same way (Table). Keto analogues of histidine,

arginine, phenylalanine and tyrosine serve as poor amino acceptor of D-amino acid aminotransferase and these amino acids were produced in a lower yield under the same conditions. However, the yields were increased to above 90% by addition of a large amount of D-amino acid aminotransferase (about 60 units). In the synthesis of D-serine, D-aspartate, and D-tryptophan a larger amount of D-amino acid aminotransferase is needed to attain the high yield.

Table. Enzymatic Synthesis of D-Amino Acids

Product	D-ATA (units)	Reaction Time (h)	Yield (%)
D-Glu	3	4	100
D-α-ABA	3	4	93
D-Norval	3	4	92
D-Met	3	4	100
D-Val	3	10	90
D-Leu	3	10	74
D-Ile	60	10	90
D-His	60	4	62
D-Arg	60	4	88
D-Phe	60	4	67
D-Tyr	60	10	100
D-Phegly	60	10	2
D-Trp	60	4	13

IV. *In situ* Determination of Stereospecificity of NAD-dependent Dehydrogenases.

Amino acid racemases inherently catalyze the exchange of α-hydrogen of amino acids with deuterium during racemization in ^2H_2O. When the reactions catalyzed by alanine racemase and L-alanine dehydrogenase, which is pro-*R* specific for the C-4 hydrogen transfer of NADH, are coupled in ^2H_2O, [4*R*-^{2}H]NADH is exclusively produced. Similarly, [4*S*-^{2}H]NADH is made in ^2H_2O with amino acid racemase with low substrate specificity (EC 5.1.1.10) and L-leucine dehydrogenase, which is pro-*S* specific. We have established a simple procedure for the *in situ* analysis of stereospecificity of C-4 hydrogen transfer of NADH by an NAD-dependent dehydrogenase by combination with either of the above two couples of enzymes in the same reaction mixture and ^{1}H-NMR analysis of the C-4 hydrogen of NAD.

V. Construction of A Chimeric NAD-Dependent Amino Acid Dehydrogenase with Broad Substrate Specificity Derived from Leucine Dehydrogenase and Phenylalanine Dehydrogenase

Amino acid dehydrogenase (EC 1.4.1.-) catalyzes the reversible deamination of amino acids to the corresponding keto acids in the presence of pyridine nucleotide coenzymes, NAD(P). We have cloned the genes of themostable leucine dehydrogenase from *Bacillus stearothermophilus* and phenylalanine dehydrogenase from *Thermoactinomyces intermedius* into *Escherichia coli*. Recombinant *E. coli* cells producing the enzymes were screened by means of a replica printing method involving a color reaction for the enzymes. The amino acid sequence of phenylalanine dehydrogenase showed 47.5% homology to that of leucine dehydrogenase. The characteristic amino acid residues conserved in the coenzyme binding domain of NAD(P)-dependent dehydrogenases were also conserved in both enzymes. The amino acid sequence similar to that of the proposed substrate binding site of glutamate dehydrogenase also occurs in both enzymes. Limited proteolysis studies showed that phenylalanine dehydrogenase is composed of two structurally dissimilar domains: the enzyme is cleaved selectively between Thr147 and Ser148 with subtilisin. The proposed substrate and NAD binding sites occur in the regions Met1-Thr147 and Ser148-Arg366-COOH of phenylalanine dehydrogenase, respectively. We have constructed a chimeric enzyme which is composed of Met1-Ser140 derived from Met1-Ser140 of phenylalanine dehydrogenase, Tyr141 and Gly142-Ile430-COOH derived from Gly141-Ile429-COOH of leucine dehydrogenase. The chimeric enzyme also was digested with subtilisin between Tyr141 and Gly142 to give two fragments. This strongly suggests that the chimeric enzyme resembles phenylalanine dehydrogenase in tertiary structure. However, the chimeric enzyme has a subunit structure of α_2 in contrast to the α_6 structure of phenylalanine and leucine dehydrogenases. The chimeric enzyme acts on not only the substrates of leucine dehydrogenase but also those of phenylalanine dehydrogenase, and shows about 6% phenylalanine dehydrogenation activity of that of the parent phenylalanine dehydrogenase.

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C-2-5

NEW FUNCTIONS OF MICROBIAL ENZYMES AND THEIR INDUSTRIAL APPLICATIONS

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ABSTRACT

D-Pantoyl lactone and D-pantoic acid are important chiral building blocks for the chemical synthesis of D-pantothenic acid. We found that *Candida parapsilosis* produces an enzyme which converts ketopantoyl lactone to D-pantoyl lactone. Similarly, conversion of ketopantoic acid to D-pantoic acid can be carried out in a better yield with the ketopantoic acid reductase of *Agrobacterium* sp. Optical resolution of DL-pantoyl lactone can be carried out with a novel fungal enzyme which specifically hydrolyzes D-pantoyl lactone to D-pantoic acid. The enzyme catalyzing this stereospecific hydrolysis has been shown to be a new lactonase. We have suggested that these enzymes can be applicable to the industrial production of D-pantothenic acid.

INTRODUCTION

In recent years, the most significant development in the field of synthetic chemistry has been the application of biological systems to chemical reactions. Reactions catalyzed by enzymes and enzyme systems display far greater specificities than more conventional forms of organic reactions, and of all the reactions available, that which has the greatest immediate potential is microbial synthesis and transformation. We have recently been carrying out studies on the development of new functions of microbial enzymes and their application for the industrial production of various biologically and chemically useful compounds -- amino acids, amides, acids, pyrogallol, theobromine, vitamins and coenzymes (Table 1).[1-6]

In this paper a recent work on "Synthesis of Chiral Intermediates for D-Pantothenate Production" is described as a typical example, with discussion focusing on what it takes to obtain better enzymes.

At present, commercial production of pantothenate depends exclusively on chemical synthesis. The conventional chemical process involves reactions yielding racemic pantoyl lactone from isobutyraldehyde, formaldehyde and cyanide, optical resolution of the racemic pantoyl lactone to D-(-)-pantoyl lactone with quinine, quinidine, cinchonidine, brucine and so on and condensation of D-(-)-pantoyl lactone with β-alanine. A problem of this chemical process apart from the use of poisonous cyanide is

Table 1. Microbial and enzymatic synthesis of useful compounds.

Product	Enzyme (source)	Yield g/L (mol%)
Amino acids		
D-*p*-Hydroxyphenylglycine	Dihydropyrimidinase (*Bacillus* sp.)	5 (74)
D-Phenylglycine	" "	6 (91)
L-Tyrosine	β-Tyrosinase (*Erwinia herbicola*)	61
L-Dopa	" "	53
L-Tryptophan	Tryptophanase (*Proteus rettgeri*)	100 (95)
L-Cysteine	Cysteine desulfhydrase (*Enterobacter cloacae*)	50 (86)
"	Cysteine synthase (*B. sphaericus*)	70 (82)
D-Cysteine	β-Chloro-D-alanine chloride-lyase (*Pseudomonas putida*)	22 (88)
L-Cystathionine	Cystathionine γ-synthase (*B. sphaericus*)	42 (92)
L-Serine	Serine hydroxymethyltransferase (*Hyphomicrobium* sp.)	52
Ethyl (*R*)-4-chloro-3-hydroxybutanoate	Aldehyde reductase (*Sporobolomyces salmonicolor*)	88 (95)
Amides and acids		
Acrylamide	Nitrile hydratase (*P. chlororaphis*)	400 (100)
"	" (*Rhodococcus rhodochrous*)	650 (100)
Methacrylamide	" (*P. chlororaphis*)	200
Crotonamide	" "	200
Nicotinamide	" (*R. rhodochrous*)	1465 (100)
Acrylic acid	Nitrilase "	380 (100)
Nicotinic acid	" "	172 (100)
6-Hydroxynicotinic acid	Hydroxylase (*Comamonas acidovorans*)	120 (96)
6-Hydroxypicolinic acid	" (*Alcaligenes faecalis*)	116 (97)
Pyrogallol	Gallic acid decarboxylase (*Citrobacter* sp.)	23 (100)
Theobromine	Oxygenase (*P. putida*)	20 (92)
D-Pantoyl lactone	Carbonyl reductase (*Candida parapsilosis*)	100 (83)
D-Pantoic acid	Aldonolactonase (*Fusarium oxysporum*)	700 (95)
Coenzymes		
Coenzyme A	Multi-step enzyme system (*Brevibacterium ammoniagenes*)	115 (95)
Adenosylmethionine	AdoMet synthetase (*Saccharomyces sake*)	12 (45)
Adenosylhomocysteine	AdoHcy hydrolase (*A. faecalis*)	74 (97)
FAD	FAD pyrophosphorylase (*Arthrobacter globiformis*)	18 (28)
Pyridoxal 5'-phosphate	PMP oxidase (*P. fluorescens*)	0.15 (98)
NADH	Formate dehydrogenase (*Arthrobacter* sp.)	30 (90)
NADPH	Glucose dehydrogenase (*Gluconobacter suboxydans*)	73 (100)
Polyunsaturated fatty acids		
Dihomo-γ-linolenic acid	Multi-step conversion (*Mortierella alpina*)	4.1
Arachidonic acid	" "	4.5
Eicosapentaenoic acid	" "	1.8

the troublesome resolution of the racemic pantoyl lactone and the racemization of the remaining L-(+)-isomer. Therefore, most recent studies in this area have concentrated on developing an efficient method to obtain D-(-)-pantoyl lactone.

To skip this resolution-racemization step, several microbial or enzymatic methods have been proposed. They are roughly divided into two types based on the starting substrate used.[1,7)]

STEREOSPECIFIC REDUCTION OF PROCHIRAL INTERMEDEATES FOR D-PANTOTHENIC ACID SYNTHESIS

Recently, we developed an efficient one-pot synthesis method for ketopantoyl lactone, in which the product is synthesized from isobutyraldehyde, sodium methoxide, diethyl oxalate and formalin (Fig. 1). The reaction is performed in one step at room temperature with a yield of 81.0%.[8] Ketopantoyl lactone is a very promising starting material for the synthesis of D-(-)-pantoyl lactone because it may permit several microbiological approaches leading to D-(-)-pantoyl lactone or D-(+)-pantothenate, as shown in Fig. 2. Thus, we assayed a variety of microorganisms as to their reducing ability using several prochiral carbonyl compounds, such as ketopantoyl lactone and ketopantoic acid.

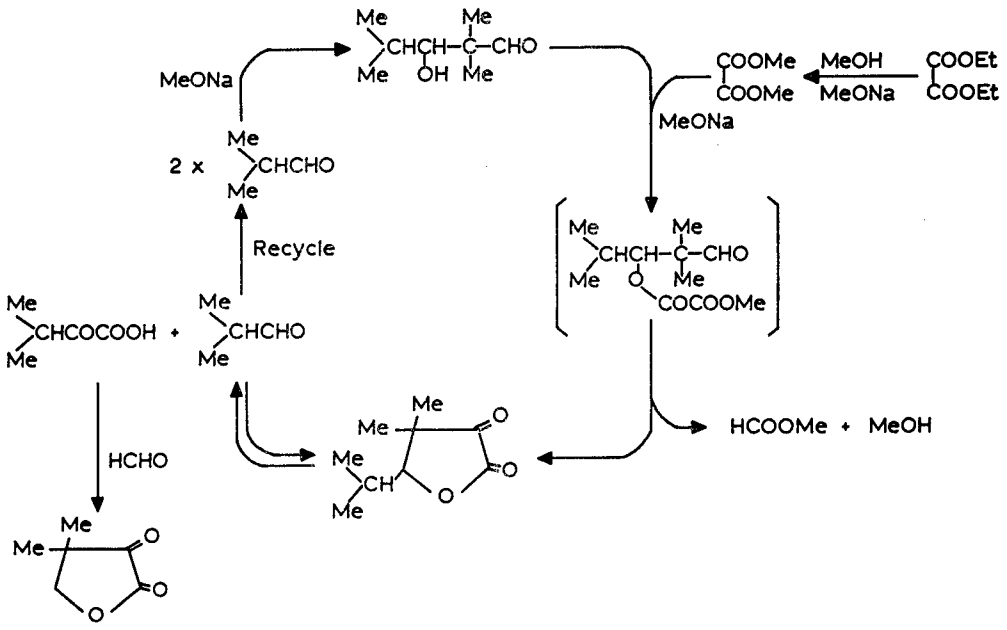


Fig. 1. The proposed reaction pathway for the one-pot synthesis of ketopantoyl lactone.

Conversion of ketopantoyl lactone to D-(-)-pantoyl lactone. This conversion (Fig. 2, reaction 1) was assayed at pH 4 to 6 by incubating ketopantoyl lactone (10 mg/ml) in the culture broth, which had been grown with each test microorganism, for 2 days at 28°C. Many microorganisms were found to convert the added ketopantoyl lactone to pantoyl lactone. However, the ratios of D- and L-isomers of formed pantoyl lactone were randomly distributed among the strains tested and the stereospecificity shown by the test strains showed almost no relation to the genera or sources.[9]

Practical stereospecific reduction of ketopantoyl lactone to D-(-)-pantoyl lactone was carried out with washed cells of *Rhodotorula minuta* or *Candida parapsilosis* as a catalyst and glucose as energy for the reduction. About 50 or 90 g/liter of D-(-)-pantoyl lactone

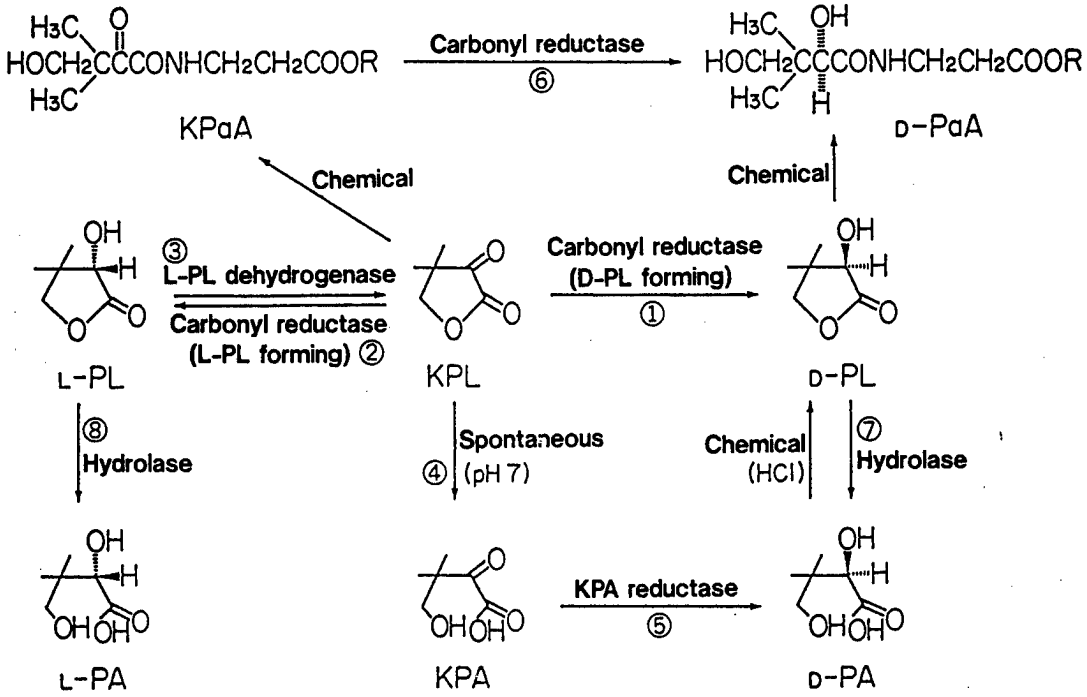


Fig. 2. Reactions involved in the enzymatic transformation to D-(-)-pantoyl lactone or D-(+)-pantothenate. D-PL, D-(-)-pantoyl lactone; L-PL, L-(+)-pantoyl lactone; KPL, ketopantoyl lactone; KPA, ketopantoic acid; D-PA, D-(-)-pantoic acid; L-PA, L-(+)-pantoic acid; KPaA, 2'-ketopantothenate; D-PaA, D-(+)-pantothenate.

(94 or 98% *ee*, respectively) was produced with a molar yield of nearly 100% by *Rhodotorula minuta* or *Candida parapsilosis*, respectively (Fig. 3).[8,10]

The enzyme catalyzing the asymmetric reduction of ketopantoyl lactone was isolated in a crystalline form from the cells of *Candida parapsilosis* and characterized in some detail[11](see also Table 2). It is a novel NADPH-dependent carbonyl reductase with a molecular mass of about 40,000. In addition to the reduction of ketopantoyl lactone, the enzyme catalyzed those of a variety of cyclic diketones, including derivatives of ketopantoyl lactone, isatin, camphorquinone and so on, and gave the corresponding *R*-alcohols.[11,12] We named the enzyme "conjugated polyketone reductase", since the enzyme catalyzes only the reduction of conjugated polyketones.[13]

Since the above-mentioned enzyme requires NADPH for the reduction of ketopantoyl lactone, there must be regeneration reaction(s) for NADPH coupled with the reduction reaction in the cells. Experiments using cell-free extracts of *Candida parapsilosis* demonstrated that hexokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were involved in the regeneration of NADPH. Cellular levels of these enzymes in *Candida parapsilosis* were almost the same as those in other common yeasts such as baker's yeast and brewer's yeast, while considerably higher activity of conjugated polyketone reductase was detected in this yeast.[14]

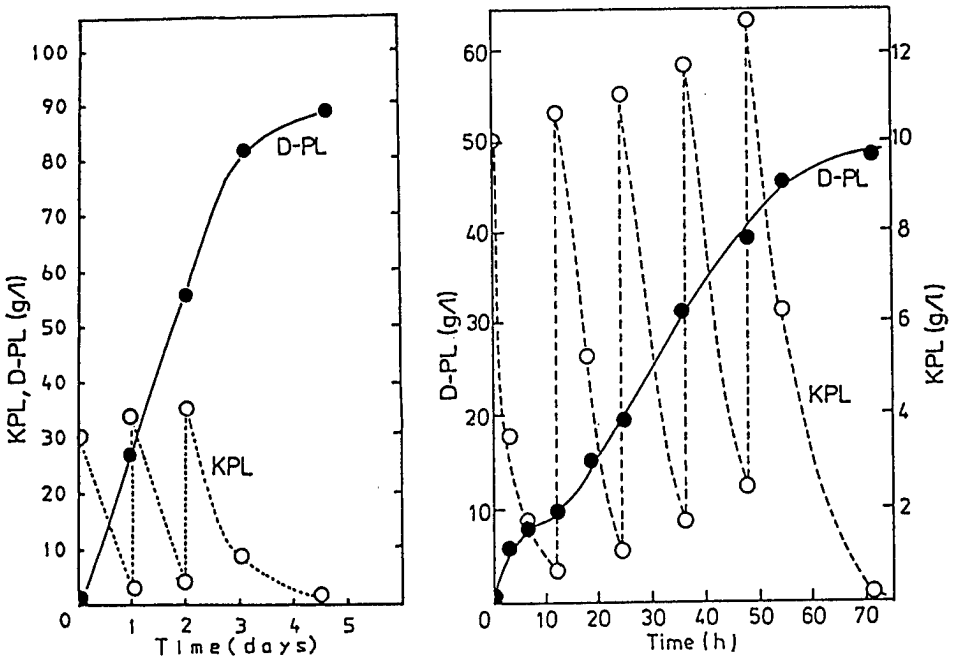


Fig. 3. Stereoselective conversion of ketopantoyl lactone (KPL) to D-(-)-pantoyl lactone (D-PL) by *Candida parapsilosis* (left) and *Rhodotorula minuta* (right).

Table 2. Properties of the polyketone reductase and ketopantoic acid reductase.

	Polyketone reductase (C.parapsilosis)	KPA reductase (P.maltophilia)		Polyketone reductase (C.parapsilosis)	KPA reductase (P.maltophilia)
Native Mr	37,000	116,000	Cofactor	NADPH	NADPH
Subunit Mr	41,600	30,500	Optimum pH	7.0	6.0
$s_{20,w}$ (S)	4.8	7.75	Optimum temp.(°C)	40	37
pI	6.3	3.5	pH stability	6.0-7.5	6.0-10
Absorption maximum (nm)	278	276	Thermal stability	42%(40°C,10min)	90%(60°C,10min)
E (1%)	8.3	20.0	Inhibitor	quercetin	-
COOH amino acid	-	Phe	Reaction mechanism	-	ordered Bi-Bi
Km (mM)	0.33 (KPL)	0.40 (KPA)	Enzyme formation	constitutive	constitutive
Vmax (μmol/min/mg)	481 (KPL)	1,310 (KPA)			

KPL, ketopantoyl lactone; KPA, ketopantoic acid.

Conversion of ketopantoic acid to D-(-)-pantoic acid. The stereospecific reduction of ketopantoic acid to D-(-)-pantoic acid by ketopantoic acid reductase (EC 1.1.1.169) (Fig. 2, reaction 5), which is involved in the pantothenate biosynthesis pathway, is also a promising reaction for the same purpose, because the ring-opening of ketopantoyl lactone to yield ketopantoic acid is easy and ketopantoic acid reductase shows absolute stereospecificity for D-(-)-pantoic acid.[15] When the same screening as described above was performed at pH 7 to 8, under which the added ketopantoyl lactone underwent rapid and spontaneous hydrolysis to ketopantoic acid, we observed a quite different

distribution profile of the reducing activity. As expected, most of the microorganisms which showed high reducing activity were found to produce only the D-isomer. Through this screening, we found that many bacteria belonging to the genus *Agrobacterium* almost specifically produce the D-(-)-isomer (>96% *ee*) in high yields. On incubation with a soil isolate, *Agrobacterium* sp. S-246, the yield of D-(-)-pantoic acid reached 119 g/liter (molar yield, 90%; optical purity, 98% *ee*).[16)]

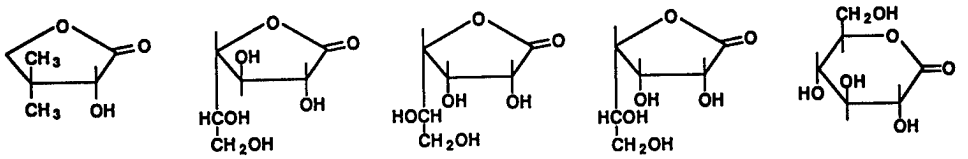
Ketopantoic acid reductase was isolated in a crystalline form from one of the potent D-(-)-pantoic acid producers, *Pseudomonas maltophila*, and characterized in some detail[15)] (see also Table 2). It is an NADPH-dependent enzyme and is strictly specific to ketopantoic acid. The observation that mutants lacking this enzyme require either D-(-)-pantoic acid or pantothenic acid for growth and that the revertants regain this activity indicates that it is involved in the pantothenate biosynthesis.

These enzymatic methods are simple and, unlike the conventional chemical resolution, require no racemization step, which is necessary for the conventional chemical resolution.

STEREOSPECIFIC HYDROLYSIS OF PANTOYL LACTONE

Kinetic resolution of DL-pantoyl lactone can be carried out by specific fungal hydrolases. We found that many mold strains belonging to the genera *Fusarium*, *Gibberella* and *Cylindrocarpon* specifically hydrolyze D-(-)-pantoyl lactone to D-(-)-pantoic acid (Fig. 2, reaction 7). On the other hand, several yeast strains hydrolyzed only the L-(+)-isomer (Fig. 2, reaction 8). For practical purposes, the former reaction is more advantageous than the latter, because, in the latter case, optical purity of the remaining D-(-)-pantoyl lactone is low unless the hydrolysis of L-(+)-pantoyl lactone is complete. Among various *Fusarium* strains tested, *Fusarium oxysporum* AKU 3702 showed the highest hydrolysis activity and gave D-(-)-pantoic acid of high optical purity (>95% *ee*). When *Fusarium oxysporum* mycelia were incubated in 70% (w/v) aqueous solution of DL-pantoyl lactone for 24 h at 30°C with automatic pH control (pH 6.8-7.2), about 90% of the D-(-)-isomer was hydrolyzed. The resultant D-(-)-pantoic acid in the reaction mixture showed a high optical purity (96% *ee*) and the coexisting L-(+)-isomer remained without any modification.[17)]

The enzyme responsible for this hydrolysis was isolated from *Fusarium oxysporum* mycelia and crystallized. It is a kind of aldonolactonase with a molecular mass of 125,000. The enzyme is composed of two identical subunits, each of which contains 1-2 mol of Ca^{2+} and about 15% carbohydrate. Ca^{2+} is necessary for the enzyme activity. Ca^{2+} also plays an important role as a stabilizer of the enzyme. Mannose is a major component of the carbohydrate. The enzyme catalyzes reversible hydrolysis of several sugar lactones, such as D-galactonolactone, L-mannonolactone, D-gulonolactone and D-gluconolactone (Fig. 4). The enzyme greatly favors the hydrolytic direction under the neutral or mild alkaline conditions. Reaction equilibrium at pH 6.0 is about 50% when D-(-)-pantoyl lactone is the substrate. Several aromatic lactones, *i.e.*, dihydrocoumarin, homogentisic acid lactone and 2-coumaranone, are also good substrates of the enzyme. All the sugar lactones which serve as substrate have a downward hydroxyl group at the 2-position, when the lactone rings are drawn according to the Haworth system. The corresponding enantiomers are competitive inhibitors.[18)]



	D-Pantoyl lactone	D-Galactonolactone	L-Mannonolactone	D-Gulonolactone	D-Gluconolactone
Relative activity (%)	100	207	264	345	221
Km (mM)	120	3.6	23	29	

Fig. 4. Substrate specificity of the aldonolactonase of *Fusarium oxysporum*.

Practical hydrolysis of the D-(-)-isomer in a racemic mixture is carried out using immobilized mycelia of *Fusarium oxysporum* as the catalyst. Stable catalyst with high hydrolytic activity can be prepared by entrapping the fungal mycelia into calcium alginate gels. When the immobilized mycelia were incubated in a reaction mixture containing 200 g/l DL-pantoyl lactone for 21 h at 30°C under the conditions of automatic pH control, 90-95% of the D-(-)-isomer was hydrolyzed (optical purity, 90-95% *ee*). After repeated reactions for 100 times (*i.e.*, 100 days), the immobilized mycelia retained more than 90% of their initail activity (Fig. 5).[19]

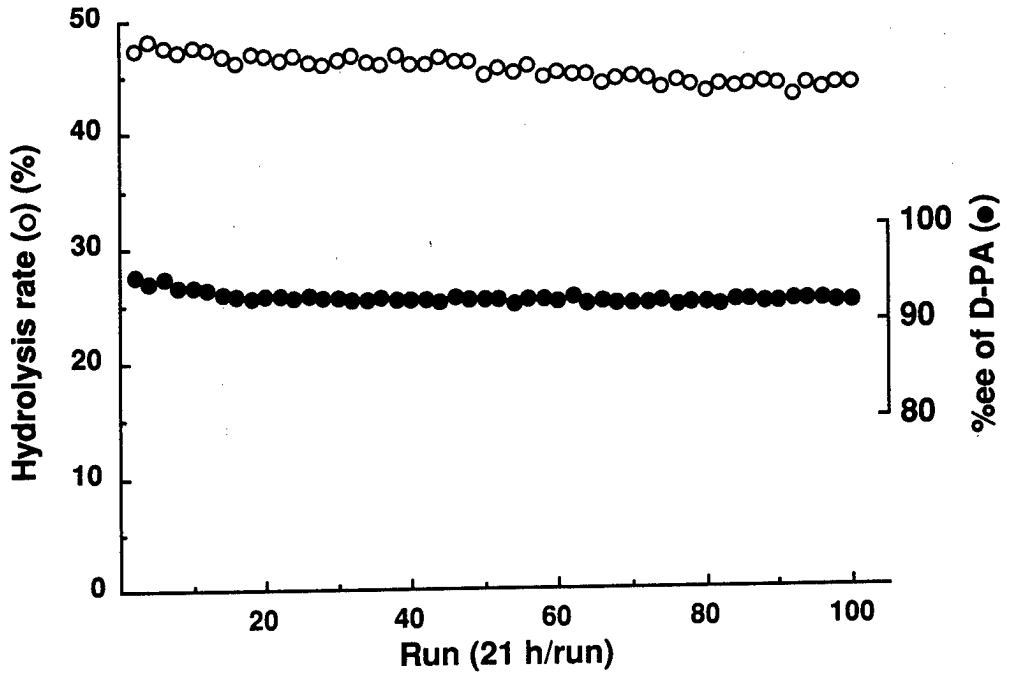


Fig. 5. Stereospecific hydrolysis of pantoyl lactone by *Fusarium oxysporum* mycelia entrapped in calcium alginate gels. D-PA, D-(-)-pantoic acid.

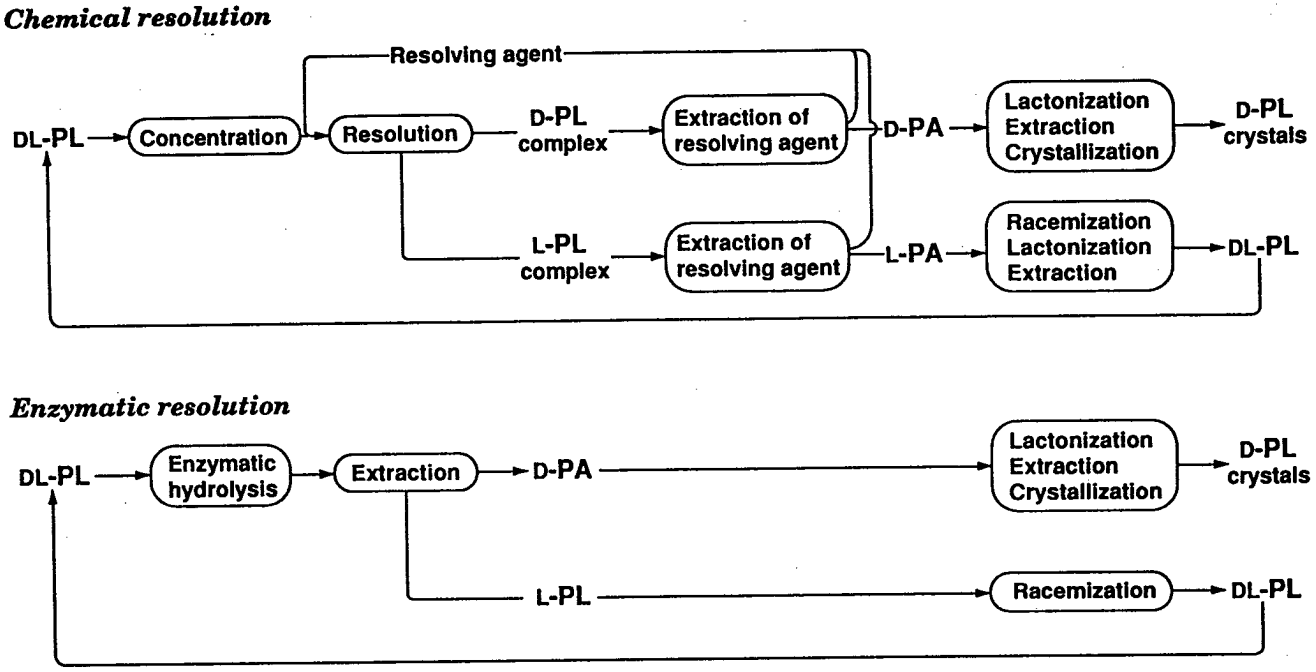


Fig. 6. Comparison of enzymatic and conventional chemical resolution processes for DL-pantoyl lactone. DL-PL, DL-pantoyl lactone; D-PL, D-(-)-pantoyl lactone; L-PL, L-(+)-pantoyl lactone; D-PA, D-(-)-pantoic acid; L-PA, L-(+)-pantoic acid.

Comparison of the enzymatic process proposed by the present study and the conventional chemical process for the resolution of DL-pantoyl lactone is shown in Fig. 6.

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MICROBIOSENSORS AND MICROMACHINES

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Abstract

Micromachining techniques were applied to construct biosensor system. The micromachined biosensors have small size, low production cost, and good reproducibility. We made some detection units for flow injection analysis (FIA). An electrochemical flow cell was fabricated, and both enzyme immobilized column and electrochemical detector were integrated onto the same chip. A chemiluminescence detector was also fabricated and applied to the quantitation of glucose and lactic acid contained in human serum and urine.

Introduction

Several kinds of microbiosensors have been developed in recent years. Since Caras and Janata [1] first reported a penicillin sensor based on an ion sensitive field effect transistor, many microbiosensors using such transducers have been developed [2,3]. Amperometric transducers, such as small oxygen electrodes [4] and integrated microelectrodes [5], have also been prepared using semiconductor fabrication technology. The small oxygen electrode has been used to make a glucose sensor, and the integrated microelectrodes to make integrated biosensors for glucose and galactose.

Recent developments and challenges in fabrication technology involve miniaturization of machines. In the last 15 years, micro fabrication techniques based on I.C. technology, such as photolithography and etching, were applied to other fields. These techniques which are used to make some small and efficient three-dimensional devices are called micromachining. For example, pressure sensors [6], accelerometers [7], micro pumps [8] and micro motors [9] were already developed using micromachining techniques.

Furthermore, some studies to make miniaturized chemical analysis systems were already reported [10,11]. These analysis systems have some advantages, such as fast response, small amount of sample, low consumption of reagents, as compared with the conventional one. On the basis of the same idea, we applied micromachining techniques to make a miniaturized enzyme-based sensor system. However, on

enzyme-based sensor system, it is a problem that the life time of enzyme is insufficient. Therefore, with the micromachined devices which can be made at the low cost, it is favorable for disposable type use.

Electrochemical Flow Cell

An electrochemical flow cell which has very small inner volume, ca. 20 nL was fabricated [12]. This flow cell can be used as an electrochemical detector for liquid chromatography or FIA. For example, a chromatogram of catecholamines using the micro electrochemical cell as a detector is shown in Figure 1. When enzyme is immobilized on the cell, the flow cell can be employed as an electrochemical biosensor.

Structure of the device

The structure of a micro electrochemical flow cell is shown in Figure 2. The 10 × 20 mm silicon chip has a V-shaped groove, whose size was 100 μm in width, 70 μm in depth, 5 mm in length. Total volume of the groove was about 20 nL. Four working electrodes, whose size was 200 μm in width, and one counter electrode, whose size was 1.5 mm in width, were formed on a PYREX glass substrate. The silicon substrate and the PYREX glass substrate were thermally bonded.

Application to electrochemical glucose sensor

Glucose oxidase (GOD) was immobilized onto the sample inlet hole of the cell using glutaraldehyde and bovine serum albumin (BSA). Figure 3 shows the measurement system. The cell was placed onto the cell holder, a plunger pump and a sample injector were connected to it. Carrier was flowed at 50 μL/min. Figure 4 shows a calibration curve for glucose. A linear relationship was obtained in the range of 30 mg/dL to 1000 mg/dL when 0.2 μL of the sample was injected. It takes about 1 minute to measure one sample. Good reproducibility was obtained using one device, however, the reproducibility was not so good between plural devices. This problem was caused by the GOD immobilization method used here. To solve this problem, we also fabricated an enzyme immobilized column with micromachining techniques.

Integration of Enzyme Immobilized Column and Electrochemical Flow Cell

A long open-tubular column was fabricated on the silicon substrate [13]. The inner wall of the column was treated with silane coupling reagent, and then enzyme was immobilized on it. This type of column has some advantages, such as low pressure drop, less diffusion, compared with conventional packed column. Moreover, an electrochemical cell whose structure is almost same as described above, was integrated on the same chip.

Structure of the integrated glucose sensor

Figure 5 shows structure of a glucose sensor which was integrated with enzyme immobilized column and electrochemical flow cell. The column was made by anisotropic silicon etching to the 100 μm in width, 70 μm in depth, 1000 mm in length, and total volume of the

column is 5 μL. Four gold electrodes were fabricated on the glass substrate. Both of the two substrates were anodically bonded. Connecting union to the pump or sample injector, was glued on inlet and outlet hole with epoxy resin. GOD was immobilized on the inner wall of the column using 3-aminopropyltriethoxysilane and glutaraldehyde. This device was put in conventional FIA system.

Response and calibration curve of the integrated glucose sensor

Figure 6 shows a typical response curve of the integrated glucose sensor when phosphate buffer (pH 7.0) as carrier was entered at the flow rate of 10 μL/min and 0.2 μL of glucose contained sample was injected. The oxidation current of the hydrogen peroxide was peaked at 1 minute after injection. A calibration curve for glucose sensor is shown in Figure 7. A linear relationship was observed at glucose concentration between 15 mg/dL and 450 mg/dL.
In case of the actual biological sample measurement, interference of other electroactive spices, such as ascorbic acid and uric acid may appear. Therefore, some separation technique or pretreatment method of the sample is needed for practical use.

Integration of Enzymatic Reactor and Chemiluminescence Detector

The flow injection analysis using chemiluminescence reaction has high sensitivity. Generally, a detector or sensor becomes small in size and the output signal becomes weak. Therefore, chemiluminescence detector is suitable for miniaturization. The luminol reaction has long been known for its use in hydrogen peroxide determination. When the enzyme reaction which generates hydrogen peroxide combines with luminol reaction, substrate can be measured by chemiluminescence. We, therefore, integrated an enzymatic reactor and a chemiluminescence detector on the same chip [14].

Structure of the integrated device

Figure 8 shows the structure of the device. It consists of a silicon and glass substrate. On the silicon substrate, an enzymatic reaction column, a mixing chamber, a spiral flow cell were made by anisotropic etching. The whole size of the measuring unit was 15 mm × 20 mm, and the total internal volume of the device was about 15 μL. Enzyme immobilized glass beads were packed into the column, and a photodiode was placed onto the spiral flow cell.

Measurement system of chemiluminescence

Enzyme was immobilized onto the glass beads (100 μm in diameter) using 3-aminopropyltriethoxysilane and glutaraldehyde. Enzyme immobilized glass beads were packed into the column on the device. Figure 9 shows the measurement system. The measuring unit was placed onto a holder in which the photodiode was involved, and three plunger pumps and a sample injector were connected. Chemiluminescence was detected by photodiode, and the signal was amplified and recorded by a pen recorder. Luminol (chemiluminescence reagent), potassium ferricyanide (catalyst of chemiluminescence reaction) and pH 7.0 phosphate buffer (carrier) were flowed by the plunger pumps individually. Buffer solution was allowed to enter at a flow rate of 20 μL/min, luminol and potassium ferricyanide were flowed at 40 μL/min and 20 μL/min, respectively. 0.2 μL of sample was injected using the sample injector.

Response and calibration curve for glucose standard solution

Using GOD immobilized glass beads, determination of glucose concentration was carried out. Figure 10 shows response curves for glucose standard solution. Good reproducibility was obtained. It takes about 1 minute to measure one sample. Figure 11 shows a calibration curve for glucose. A linear relationship was obtained in the range of 10 mg/dL to 300 mg/dL.

Quantitation of glucose contained in human serum and urine

Glucose contained in human serum and urine was quantitated by chemiluminescence detector. Glucose was added to the controlled human serum (Wako Pure Chemical Industries, LTD., control serum WAKO, normal and abnormal) and controlled human urine (Bio-Rad Laboratories, Liphocheck quantitative urine control), to adjust the glucose concentration between 10 mg/dL - 350 mg/dL. These samples were injected with no pretreatment, apart from filtration. For a reference, the samples were also quantitated by clinical inspection kit (Wako Pure Chemical Industries, LTD., Glucose Test Wako B, GOD - POD method). Correlation coefficient between this chemiluminescence method and the conventional method was 0.99.

Quantitation of lactic acid contained in human serum

Lactic acid contained in human serum was quantitated using the same procedure as the glucose determination. For a reference, the samples were also quantitated by Boehringer Manheim, F-kit for L-lactic acid. The sample containing L-lactic acid at the concentration from 4 mg/dL to 50 mg/dL can be measured. Correlation coefficient between this chemiluminescence method and the conventional method was 0.98.

Conclusion

The detection units for enzyme-based FIA were fabricated using micromachining techniques. These micromachined devices are batch-processed, therefore, these can be made at low cost, and have good reproducibility. The signal from the detector becomes weaker as its size becomes smaller. However, the electrochemical or chemiluminescence method is more sensitive than spectroscopic method, therefore, the measurable range of the micromachined detector is almost same as the conventional method.
Additionally, the measurement can be carried out at the flow rate of reagent less than 50 µL/min and then the pressure drop on these devices, except the enzyme immobilized column, was less than 0.1 atm. These data indicate the possibility of applying the micromachined pump. Thus, the conventional plunger pump and sample injector used in the above-mentioned experiments will be replaced by micromachined devices in the future.

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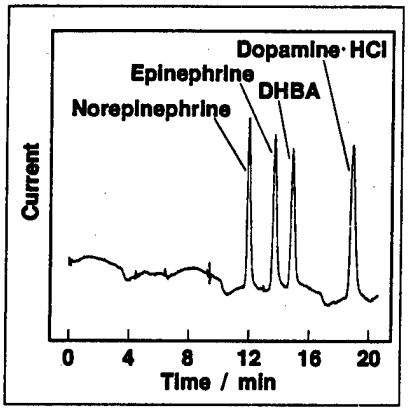

Figure 1. Chromatogram of catecholamines using electrochemical flow cell; column = Beckman Ultrasphere I.P. C-18, 4.6 mm × 250 mm; eluent = 75 mmol/L NaH_2PO_4, 1 mmol/L 1-octanesulfonic acid sodium salt, 50 μmol/L EDTA-2Na, 11% acetonitrile; flow rate of the eluent = 0.3 mL/min; concentration of catecholamines = 100 μmol/L.

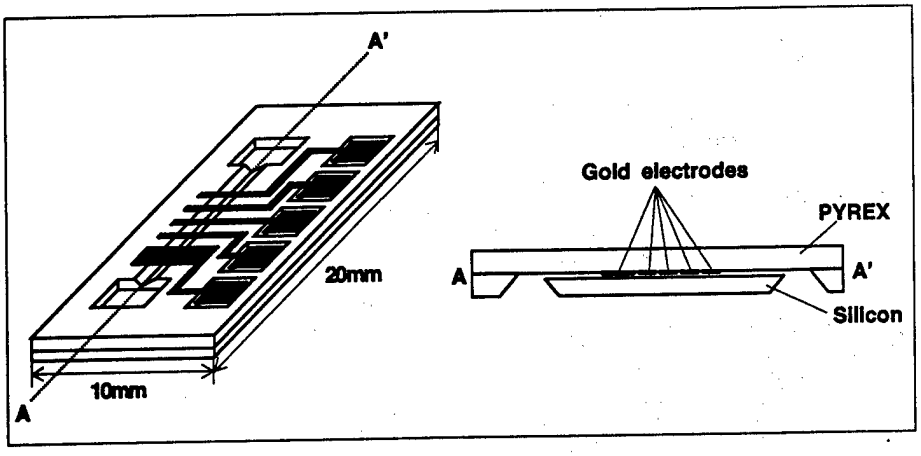

Figure 2. Structure of the micro electrochemical flow cell.

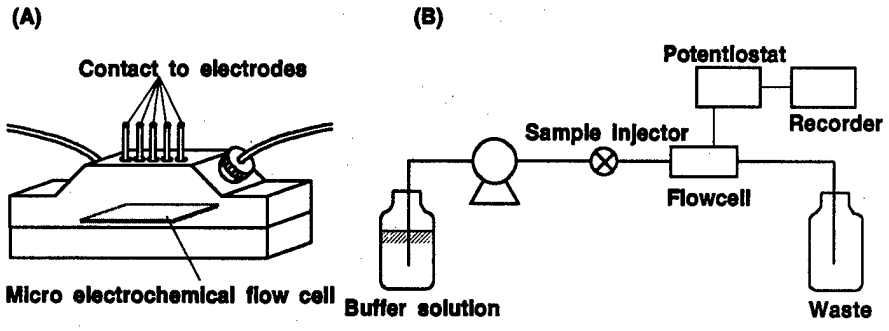

Figure 3. Measurement system; (A) Structure of the flow cell. (B) Schematic diagram of the system.

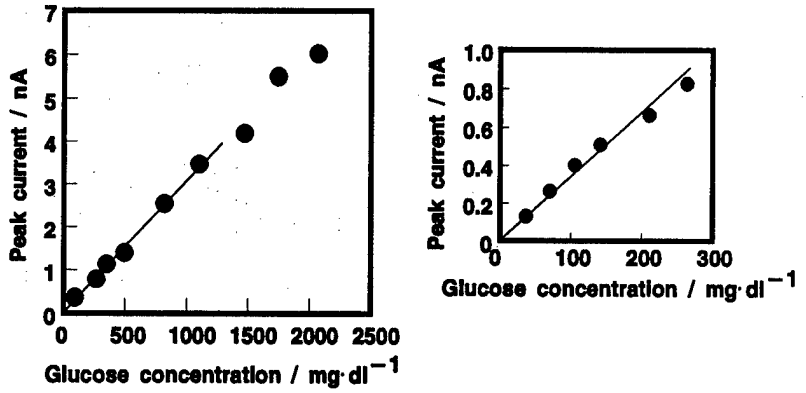

Figure 4. Calibration curve for glucose. Measurement was carried out at carrier (0.1 mol/L phosphate buffer, pH 7.0) flow rate = 50 µL/min; sample volume = 0.2 µL.

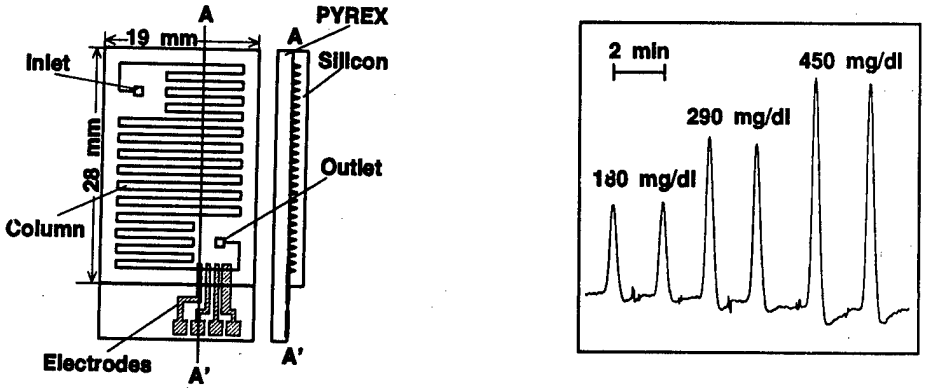

Figure 5. Structure of the integrated glucose sensor.

Figure 6. Response curves of the detector. Measurement was carried out at carrier (0.1 mol/L phosphate buffer, pH 7.0) flow rate = 10 µL/min; sample volume = 0.2 µL.

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